The Role of $CD4^+$ T Cells in Friend Virus Infection

Rebecca St Clair Pike

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Supervisor: Dr George Kassiotis

Division of Immunoregulation

MRC National Institute for Medical Research

Mill Hill, London NW7 1AA

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I, Rebecca St Clair Pike, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Retroviruses can establish persistent infection despite induction of a multipartite antiviral immune response. Whether collective failure of all parts of the immune response or selective deficiency in one crucial part underlies the inability of the host to clear retroviral infections is currently uncertain. In this study, the contribution of virus-specific $CD4^+$ T cells in resistance against Friend virus (FV) infection in the murine host is examined. The results show that the magnitude and duration of the FV-specific $CD4^+$ T cell response is directly proportional to resistance against acute FV infection and subsequent disease. Notably, significant protection against FV-induced disease is afforded by FV-specific $CD4^+$ T cells in the absence of a virus-specific $CD8^+$ T cell or B cell response. Enhanced spread of FV infection in hosts with increased genetic susceptibility causes a proportional increase in the number of FV-specific $CD4^+$ T cells required to control FV-induced disease. Thus, these results suggest that FV-specific $CD4^+$ T cells provide significant direct protection against acute FV infection, the extent of which critically depends on the ratio of FV-infected cells to FV-specific $CD4^+$ T cells. In addition to precursor frequency, the T cell receptor (TCR) affinity for antigen is also considered an important contributor to $CD4^+$ T cell expansion and effector function. Study of polyclonal TCRβ-transgenic FV-specific $CD4^+$ T cells has previously revealed a distinct pattern of clonal evolution during FV infection. In the current study, immunisation regimens, including adjuvanted peptide and replication-attenuated retrovirus vaccination, are assessed for the overall TCR affinity of the $CD4^+$ T cell population they elicit. The implications for vaccine design and vaccine-induced $CD4^+$ T cell-mediated protection are discussed.

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Materials and Methods

List of Abbreviations

AB	Air-buffered
Ab	Antibody
ACK	Ammonium-chloride-potassium
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cells
B6	C57BL/6
BM	Bone Marrow
CCR	Chemokine (C-C motif) Receptor
cDNA	Complementary DNA
CFA	Complete Freunds Adjuvant
CFSE	Carboxylfluorescein Succinimidyl Ester
CTL	Cytotoxic Lymphocyte
CXCL	Chemokine (C-X-C motif) Ligand
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
D-PBS	Dulbecco's Phosphate Buffered Saline
dsRNA	Double-Stranded Ribonucleic Acid
EBV	Epstein-Barr Virus
env	Envelope

EpoR	Erythropoeitin Receptor
FACS	Fluorescence Activated Cell Sorter
FcR	Crystallisable Fragment Receptor
F-MuLV	Friend Murine Leukaemia Virus
FoxP3	Forkhead Box P3
FV	Friend Virus
GFP	Green Fluorescence Protein
glyco-Gag	Glycosylated Product of the Viral gag Gene
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
i.p.	Intra-peritoneal
i.v.	Intravenous
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
LCMV	Lymphocytic Choriomeningitis Virus
LDV	Lactate Dehydrogenase-Elevating Virus
LN	Lymph Node

LPS	Lipopolysaccharide
LTNP	Long-Term Non-Progressor
mAb	Monoclonal Antibody
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
nAb	Neutralising Antibody
NK	Natural Killer
NTP	Nucleoside Triphosphate
PBS	Phosphate Buffered Saline
PIC	Pre-integration Complex
PRR	Pattern Recognition Receptor
RAG	Recombination Activating Gene
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Standard Error of the Mean
SFFU	Spleen Focus-Forming Units
SFFV	Spleen Focus-Forming Virus
SI	Spleen Index
SIV	Simian Immunodeficiency Virus
SP	Spleen

T_{CM}	Central Memory T cell
TCR	T Cell Receptor
T_{EM}	Effector Memory T cell
TGF-β	Transforming Growth Factor β
Th	T Helper Type Cell
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
VSV	Vesicular Stomatitis Virus
WT	Wild-Type

Chapter One

1 Introduction

1.1 The Innate Immune System

The mammalian immune system comprises the fast-acting, non-specific innate branch and the slower acting, pathogen-specific adaptive branch. When a pathogen infects a host, the innate and adaptive elements of the immune response work in synergy to control or, if successful, clear the infection, while simultaneously avoiding damage to the host.

The innate immune system consists of physical and chemical barriers, innate immune lymphocytes, and the complement system. Physical barriers such as the epidermis and chemical barriers such as gastric acid provide the initial defence against invasion by a pathogen, while complement and innate immune cells are recruited if a pathogen is successful in evading these initial barriers.

1.1.1 Mononuclear phagocytes

Mononuclear phagocytes act by engulfing and destroying a pathogen or infected cell, and include macrophages and neutrophils. These cells are activated by cell damage or via interactions between pathogenic components and innate immune receptors called pattern recognition receptors (PRRs). Although their major role is phagocytosis, they also produce microbicidal reactive oxygen species (ROS) and nitric oxide (NO), and release chemokines and cytokines (Gordon, 2007).

1.1.2 Natural killer cells

Natural killer (NK) cells are large granular lymphocytes which comprise around 10-15% of all peripheral blood lymphocytes. They do not have antigen-specific receptors but can recognize and kill abnormal cells such as virally-infected cells and tumour cells through a range of activating and inhibitory receptors, for example NKG2D and KIR respectively (Lanier, 2008). NK cell activity is induced by a range of cytokines, such as type I interferon (IFN) including IFN-α and IFN-β, to produce effector cytokines such as TNF-α and the type II IFN IFN-γ, mediate antibody-dependent cellular cytotoxicity (ADCC), and directly kill virus-infected cells via production of perforin and granzymes (Vivier et al., 2008). More recently, and somewhat out of character for cells of the innate immune system, NK cells have been shown to have memory capacity (Sun et al., 2010).

1.1.3 Other innate immune cells

Basophils, eosinophils and mast cells are immune mediators of type 2 immune responses against infection with parasites such as *Schistosoma mansoni*. Basophil differentiation is driven by IL-3, and they produce the type 2 cytokines IL-13, and IL-4 which promotes Th2 $CD4^+$ T cell responses, as well as lipid mediators in order to promote vascular permeability. Eosinophils are activated by IL-3 and IL-5, are predominantly localised to the mucosal tissues and produce a range of cytokines and other non-cytokine effector proteins. Mast cells produce histamine, among other chemical mediators, and cytokines including IFN-γ and TNF-α, and have major roles in both the immune response to pathogens and allergic hypersensitivity reactions (Stone et al., 2010).

1.1.4 Toll-like receptors

An important type of PRR involved in the induction of the immune response is the family of toll-like receptors (TLRs), which were initially described in *Drosophila* and later in humans (Medzhitov et al., 1997). TLRs are expressed on the surface of innate immune cells and are able to non-specifically recognise components of invading pathogens, known as pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) and double stranded ribonucleic acid (dsRNA), from bacteria and viruses respectively (Medzhitov and Janeway, Jr., 1997). TLR agonists can be used as adjuvants in vaccine preparations to facilitate uptake, processing and presentation of antigen which otherwise has low immunogenicity (van et al., 2006).

1.1.5 The complement system

The complement system comprises many proteins, some of which are enzymes, that make the components of the complement cascade which leads to inflammation, cell opsonisation and thus cell killing, and ultimately formation of the terminal complement complex (TCC), resulting in the lysis of a cell. As well as being initiated by innate immune receptors, complement can also be initiated by the adaptive immune system via antibody (Ricklin et al., 2010).

1.1.6 Innate cytokines

Cytokines produced during the innate immune response are required to activate and recruit other innate immune cells, as well as to have effects on the proliferation and function of adaptive immune cells. The major innate cytokines produced during the early inflammatory stages of the innate immune response are tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and transforming growth factor-β (TGF-β), which together cause inflammation, coagulation and fever in the host. In addition, these cytokines lead to expression of adhesion molecules such as selectins, and the production of chemokines such as CXCL8 to enable recruitment of other phagocytic cells to the site of infection (Biron, 1998).

Another important family of cytokines in the innate immune response are the type I IFNs IFN-α and IFN-β, which are produced mainly by plasmacytoid dendritic cells (DCs), and are induced during viral infections (Theofilopoulos et al., 2005). Type I IFNs have a range of functions which bridge the innate and adaptive immune responses, for example inducing production of innate inflammatory cytokines such as TGF-β and TNF-α, as well as inducing production of the type II interferon IFN-γ by the T cells of the adaptive immune response (Biron, 2001). Signals from IFN-α and IFN-β also lead to increased expression of major histocompatibility (MHC) class I molecules and co-stimulatory molecules on antigen presenting cells (APCs), enhancing antigen presentation to $CD8^+$ T cells, and are able to enhance cytotoxic activity of NK cells, increasing clearance of a pathogen (Biron, 2001; Biron et al., 1999).

IL-12 is produced by innate cells including macrophages and dendritic cells and is induced in the host in response to infections with many pathogens including *Listeria monocytogenes* (Hsieh et al., 1993; Macatonia et al., 1995), Leishmaniasis (Scharton-Kersten et al., 1995), as well as viral infections (Orange and Biron, 1996; Biron and Gazzinelli, 1995). The major functions of IL-12 are activation of macrophages and induction of IFN-γ production by NK cells, which mediates many downstream effects (Biron and Gazzinelli, 1995). Additionally, IL-12 drives the differentiation of $CD4^+$ T helper cells to a specific phenotype (Hsieh et al., 1993).

IFN-γ has diverse functions in both the innate and adaptive branches of the immune response and connects the two facets of the immune response. As a part of the innate immune response IFN-γ, produced primarily by NK cells, increases antigen presentation by upregulating MHC class I and II expression on APCs, activates macrophages, and induces anti-viral gene expression as well as driving the generation of an anti-viral state (Schroder et al., 2004). IFN-γ also has important roles during the adaptive immune response which will be discussed in the context of $CD4^+$ T helper cell function in section **1.3.2**.

1.2 The Adaptive Immune System

The T cells and B cells of the adaptive immune system are derived from a common lymphoid progenitor in the bone marrow, and while B cells continue to develop in the bone marrow, T cells migrate to and develop in the thymus. Both T cells and B cells enter and circulate the peripheral blood and secondary lymphoid organs as naive cells until they encounter antigen presented to them by APCs, and become activated to proliferate and contribute towards the control of infection via a range of mechanisms. While T cells recognise antigen via their T cell receptor (TCR), B cells recognise antigen via their B cell receptor (BCR).

1.2.1 T cells

T cells initially develop in the thymus as $CD4^+CD8^+$ co-receptor double-positive precursors, but subsequently commit to the CD4 or CD8 lineage, becoming either T helper cells or cytotoxic T cells respectively (Hernandez-Hoyos et al., 2003). T cells further undergo clonal selection in the thymus, where cells possessing self-reactive TCRs experience high affinity interactions with self-antigen and are deleted, while those that experience low affinity interactions with self-antigen survive (Klein et al., 2009). While $CD8^+$ cytotoxic T cells act by directly killing infected cells, $CD4^+$ T cells are helper cells which act by producing cytokines to promote $CD8^+$ T cell and B cell function.

$CD8^+$ T cells are important for control and elimination of intracellular infections and cancerous tumours. $CD8^+$ T cells are able to directly kill infected cells via several mechanisms. The major mechanism of $CD8^+$ T cell-mediated cytotoxicity is release of

perforin to generate a pore in the target cell membrane, allowing administration of caspases known as granzymes which consequently break down intracellular proteins causing apoptosis of the cell. An alternative mechanism by which $CD8^+$ T cells can kill infected cells or tumour cells is via ligation of Fas on target cells resulting in target cell death (Masopust et al., 2007). The major role of $CD4^+$ T cells is to produce cytokines which activate this $CD8^+$ T cell mediated killing, and to induce B cell activation and function. The effector mechanisms of $CD4^+$ T cells will be discussed in more detail in section **1.3.2**.

1.2.2 Antigen presentation

APCs present antigen after they are infected with a pathogen, or after ingesting pathogens or infected cells. Antigen is presented in complex with MHC molecules, and recognised by the TCR expressed on $CD4^+$ or $CD8^+$ T cells, which determine the antigen-specificity of a T cell. Co-stimulatory signals are also required for activation of T cells, and these are transmitted through ligation of CD28 on the T cell with co-stimulatory molecules on the APC such as CD80 (B7-1) or CD86 (B7-2).

While $CD8^+$ T cells recognise antigen via their TCR in the context of MHC class I, $CD4^+$ T cells recognise antigen in the context of MHC class II. The MHC class II molecule is heterodimeric, comprising an α and β chain, and the peptide binding cleft of this MHC molecule is created by a groove formed by arrangement of the three-dimensional structure (Bjorkman et al., 1987). It is in this binding cleft that peptide binds and is presented to a T cell to induce antigen-specific proliferation. While

dendritic cells (DCs) are the main professional APC, macrophages and B cells also express MHC class II and so are able to present antigen to $CD4^+$ T cells.

1.2.3 γδ T cells

While conventional $CD4^+$ and $CD8^+$ T cells have a heterodimeric αβ TCR, another described subset of T cells have a heterodimeric γδ TCR, and are known as γδ T cells. These cells develop in both the thymus and periphery, and are known as innate-like lymphocytes due to their innate phenotype and function (Born et al., 2006). They are mainly found in the epithelium, and a major role of these cells is as a part of the initial defence against infection at mucosal surfaces, for example herpes simplex virus type 2 (HSV-2) (Nishimura et al., 2004).

γδ T cells have diverse roles, and have been shown to expand in animal models of viral, bacterial and parasitic infections, and are able to produce the required cytokines for control of these pathogens. They also have a role in tumour immune surveillance and autoimmune disease (Carding and Egan, 2002). As regards innate function, as well as their TCR, γδ T cells can express receptors typically found on innate cells, including TLRs and NK cells receptors, and unlike αβ T cells they can also act as APCs (Born et al., 2006; Moser and Brandes, 2006). However, γδ T cells also have a role in the adaptive immune response, and can produce type 1 and type 2 cytokines, as well as influencing the phenotype of responding $CD4^+$ T cells. They have also been shown to produce IL-17, particularly during bacterial infections (Roark et al., 2008).

1.2.4 B cells

Unlike T cells, which recognise antigen via their TCR in the form of peptide:MHC complexes on the surface of APCs, B cells recognise soluble antigen via their B cell receptor (BCR), leading to differentiation of B cells into Ab-secreting plasma cells and hence induction of the humoral immune response. Antibodies mediate many activities against pathogens including virus-neutralisation, mediation of Ab-dependent cellular cytotoxicity (ADCC), and activation of the complement system. Long-lived plasma cells circulate in the peripheral blood and constantly produce neutralising Ab (nAb) in case of a secondary infection (Slifka et al., 1998). B cells also play a role in controlling homeostasis of T cells, and in particular $CD4^+$ T cells (LeBien and Tedder, 2008).

B cells can produce inflammatory cytokines, while a subset of cells, known as Breg cells, are immunoregulatory and produce the immunosuppressive cytokine IL-10. Dysregulated B cells also contribute to autoimmunity, for example via production of autoantibodies, which contribute to pathogenesis of chronic inflammatory conditions including rheumatoid arthritis and systemic lupus erythematous (SLE) (Lund and Randall, 2010).

1.2.5 Immunological memory and vaccination

After the primary immune response against a pathogen, the population of antigen-specific adaptive immune cells undergoes contraction, with a small number (<10%) persisting as pathogen-specific memory cells. This occurs in both antigen-specific T cell and B cell pools, and in the case of reinfection, these pathogen-specific memory lymphocytes mount a secondary response which is more rapid and effective than that of the primary response, and works to prevent disease or accelerate pathogen clearance (Sallusto et al., 2010). This is defined as immunological memory, and is a remarkable outcome of mammalian evolution.

This ability of the immune system to develop immunological memory has been exploited in the use of vaccination, where an immune response is artificially induced in the host leading to the development of memory, and thus priming the immune system for subsequent challenge so that in the event of exposure to the pathogen which the individual has been vaccinated against, a secondary immune response can be rapidly mounted to prevent establishment of infection. Types of vaccination in use or currently being developed include live-attenuated virus vaccines, subunit vaccines, inactivated virus particles, viral vectors and DNA vaccines. Often an adjuvant is utilised to enhance the immunogenicity of a vaccine, for example, MF59 which is used successfully in seasonal influenza vaccines (Black et al., 2010; Pellegrini et al., 2009). Vaccine regimens also vary, with some vaccines requiring boosters while others provide life-long protection after one inoculation.

Vaccination programmes have been successful in completely eradicating some diseases, for example smallpox, where antibodies to the virus are still circulating in recipients of the vaccine up to 75 years later (Hammarlund et al., 2003). However, for some pathogens, a vaccine which induces a protective memory immune response is currently unavailable.

Pathogen-specific nAb can immediately neutralise a pathogen in the case of reinfection, and subsequently memory B cells and T cells expand and mount their secondary response (Sallusto et al., 2010). Hence, vaccines which induce nAb provide a more protective memory response than those inducing T cell responses.

1.3 $CD4^+$ T cells

The primary function of $CD4^+$ T cells is to produce cytokines to provide immunological help to other cells of the adaptive immune system, assisting $CD8^+$ T cells to kill virally infected cells, and B cells to produce Ab via the release of cytokines. The importance of $CD4^+$ T cells in the immune response is evident in primary and secondary immunodeficiencies. Patients suffering from bare lymphocyte syndrome, who have an MHC class II deficiency and hence a $CD4^+$ T cell deficiency, have impaired humoral and cytotoxic T cell responses. As such, they are extremely susceptible to viral, bacterial, fungal and protozoal infections such as *Pseudomonas*, *Salmonella* and *Escherichia* species, cytomegalovirus (CMV) and *Candida*, generally leading to death during childhood (Klein et al., 1993).

Infection of humans with human immunodeficiency virus (HIV) causes progressive depletion of $CD4^+$ T cells, inevitably resulting in Acquired Immunodeficiency Syndrome (AIDS), where the host succumbs to fatal opportunistic infections such as *Pneumocystis jiroveci* and cancers such as Kaposi's sarcoma. It is evident from these conditions that $CD4^+$ T cells are a vital component of the mammalian immune response.

1.3.1 $CD4^+$ T cell subsets

In 1989, two T helper cell clones with different cytokine profiles were identified and designated Th1 and Th2. It was subsequently realised that $CD4^+$ T cells differentiate into effector cells of different phenotypes upon encounter with antigen (Mosmann and Coffman, 1989).

The fate of a $CD4^+$ T cell is determined by the cytokines present during differentiation with IL-12 activating signal transducer and activator of transcription (STAT) 4 directing cells towards a Th1 phenotype, and IL-4 activating STAT6 directing cells towards a Th2 phenotype. The Th1 and Th2 subsets can be defined by their production of IL-2 and IFN-γ, or IL-4, IL-5 and IL-13 respectively. In turn, production of IFN-γ by Th1 cells inhibits differentiation of Th2 cells, and production of IL-4 by Th2 cells inhibits the differentiation of Th1 cells (Fernandez-Botran et al., 1988). Th1 and Th2 cells also produce the suppressive cytokine IL-10 as a self-regulatory mechanism, resulting in a negative feedback effect on production of effector cytokines by the cell (Moore et al., 2001). Functionally, Th1 cells have been implicated in responses against intracellular infections such as *Leishmania* and *Toxoplasma gondii*, as well as autoimmune disease (Murphy and Reiner, 2002; Paul and Seder, 1994). Conversely, Th2 cells help to control extracellular parasitic infections, for example helminths, and are associated with control of the humoural immune system. They also play a role in allergic reactions when dysregulated (Faulkner et al., 1998; Soussi-Gounni et al., 2001).

The more recently described Th17 $CD4^+$ T cells are polarised by IL-6. Th17 cells have been shown to help protect against bacterial extracellular infections such as *Klebsiella pneumoniae* and *Salmonella enterica*, fungal infections such as *Candida albicans*, and intracellular bacterial infections such as *Mycobacterium tuberculosis* via production of IL-17, IL-22 and IL-23 but also play a role in autoimmune disease (Curtis and Way, 2009). Mice deficient in Th17 cytokines or their receptors are resistant to Experimental Autoimmune Encephalomyelitis (EAE), a murine model of multiple sclerosis, while in the human autoimmune disease SLE, patients produce significantly elevated levels of IL-17 and IL-23 (Stockinger and Veldhoen, 2007; Wong et al., 2008).

T follicular helper $CD4^+$ T cells (Tfh) specifically provide help to B cells in an antigen-specific manner in the B cell follicle in the lymphoid nodes. IL-21 is the major effector cytokine of Tfh cells and plays a role in development of germinal centres and antibody isotype switching, for example from IgM to IgG. IL-21 also stimulates differentiation of Tfh cells in an autocrine manner. These cells predominantly differentiate from other Th cell subsets and can be defined by their expression of C-X-C chemokine receptor 5 (CXCR5), which directs them to the B cell follicle (Fazilleau et al., 2009).

Regulatory T (Treg) cells comprise 5-10% of all $CD4^+$ T cells and act to regulate immune responses, preventing immunopathology via production of immunosuppressive cytokines (Sakaguchi, 2004). The major effector cytokine of Treg cells is IL-10, but IL-35 has also been shown to be produced by Tregs and to be

inhibitory to other immune cells (Collison et al., 2007). The majority of Treg cells develop in the thymus but an inducible Treg (iTreg) cell population has recently been identified. Treg cells which develop in the thymus can be distinguished from iTreg, which undergo conversion from naive non-Treg $CD4^+$ T cells in the presence of TGF-β in the periphery, as natural Treg (nTreg) cells (Quintana and Cohen, 2008).

As well as different cytokine profiles, $CD4^+$ T cell subsets can be defined by expression of unique subset-specific transcription factors, with Th1 cells expressing T-bet, Th2 cells expressing GATA3 and Th17 cells expressing RORγt (Ho and Glimcher, 2002; Ivanov et al., 2006). Treg cells can be defined by expression of CD25 and the Foxp3 transcription factor (Fontenot et al., 2003), while Tfh can be defined by expression of Bcl-6 (Fazilleau et al., 2009).

Th cell subsets were initially identified in $CD4^+$ T cell clones *in vitro* but these concepts have also been observed *in vivo*. In influenza infection, mice infected intravenously develop a response dominated by Th1 $CD4^+$ T cells while mice infected intra-nasally develop a predominantly Th17 $CD4^+$ T cell response. This is attributed to differentiation of naive $CD4^+$ T cells in the presence of IL-12-producing splenic DCs after intravenous infection, while after intra-nasal infection responding $CD4^+$ T cells differentiate in the presence of IL-6-producing mucosal DCs (Pepper et al., 2010).

Despite the ongoing description of new T helper cell subsets, more recently it has been accepted that there is a degree of plasticity between the different subsets, and

that once a naive cell has differentiated into a certain subset it is not necessarily committed for its whole lifespan and, under certain environmental conditions, can convert to an alternative subset. For example, Veldhoen et al. showed that the presence of TGF-β can cause Th2 cells to convert to an IL-9 producing subset *in vitro*. This IL-9 producing subset is distinct as it does not express any of the transcription factors found in other $CD4^+$ T cell subsets, and was provisionally designated as the Th9 subset (Veldhoen et al., 2008). *In vivo,* Th17 cells specific for a pancreatic islet antigen were transferred into mice and seen to convert into cells with a Th1 effector function, inducing diabetes via IFN-γ production (Bending et al., 2009). Furthermore, in LCMV infection adoptively transferred Th2 cells were shown to reprogram to a subset with both Th1 and Th2 functions, as well as expressing both T-bet and GATA-3 transcription factors. This subset was able to be maintained for several months *in vivo* and, in the presence of IL-12 amongst other factors, induction of type I and type II interferons by lymphocytic choriomeningitis virus (LCMV) infection was essential for this reprogramming (Hegazy et al., 2010). More recently it has been shown that the mechanism by which commitment to the Th2 lineage is reversed in humans involves suppression of the GATA3 transcription factor by type I IFNs (Huber et al., 2010).

In summary, it is now acknowledged that $CD4^+$ T cells possess a certain degree of plasticity and versatility, and are able to modify their function in order to mount the most appropriate response to a pathogen.

1.3.2 Effector functions of $CD4^+$ T helper cells

IL-2 and IL-4 are autocrine growth factors for Th1 and Th2 cells respectively. $CD4^+$ T cells are the main producers of IL-2 and, upon antigen stimulation, they upregulate the IL-2 receptor (IL-2R) and hence are able to receive more signals from IL-2 to promote clonal expansion of the antigen-specific population. In Th1 $CD4^+$ T cells, IL-2 promotes synthesis of IFN-γ and further IL-2. It is also able to stimulate B cell growth and promote antibody synthesis, and stimulate growth and cytolytic activity of NK cells (Waldmann, 2006).

IFN-γ produced by Th1 cells has a broad range of anti-viral effects, including stimulating expression of MHC class I and II, activating APCs and increasing expression of co-stimulatory molecules on DCs, further augmenting antigen presentation. It also plays an important role in helping $CD8^+$ T cells to kill virally infected cells via perforin and granzymes, and can enhance the microbicidal phagocytic activity of macrophages. In a more direct fashion IFN-γ can inhibit viral replication via induction of the anti-viral enzyme PKR (Schroder et al., 2004). This effect has been shown *in vitro* against hepatitis C virus (HCV) in concert with TNF-α, and in γ-herpesvirus infection in B cell-deficient mice (Christensen et al., 1999; Davis et al., 2008).

The Th2 cytokines IL-4 and IL-5 mediate the response to extracellular infections such as parasites (Finkelman et al., 1997). IL-5 directly activates eosinophils and promotes their growth and differentiation, while IL-4 plays a major role in Ig heavy chain class switching of antibody from IgG to the IgE isotype. IgE binds to Fc receptors on cells

and has a range of effects, for example stimulating degranulation of mast cells (Stone et al., 2010).

Several recent studies have highlighted a previously unappreciated role for Tfh cell-derived IL-21 in chronic viral infection. Although absence of IL-21 during acute LCMV infection does not affect viral load or priming of LCMV-specific $CD8^+$ T cells, lack of IL-21 signalling during chronic LCMV infection leads to a reduced number and polyfunctionality of $CD8^+$ T cells, resulting in a higher viral titre (Elsaesser et al., 2009; Frohlich et al., 2009; Yi et al., 2009). Elevated numbers of IL-21-producing $CD4^+$ T cells were also seen during the chronic phase of LCMV infection, with more IL-21 produced per $CD4^+$ T cell. Furthermore, treatment with IL-21 was able to restore $CD8^+$ T cell survival and function and reduce viral titre in chronically infected *Il21r*$^{-/-}$ mice (Yi et al., 2009).

1.3.3 Cytotoxic activity of $CD4^+$ T cells

As well as providing immunological help via production of cytokines, $CD4^+$ T cells have been shown to have direct anti-viral effects, with MHC class II-dependent killing identified in several infections, such as influenza (Maimone et al., 1986). These cytotoxic $CD4^+$ T cells were initially only documented *in vitro* against pathogens such as EBV, but later studies showed that they are also active *ex vivo* in certain diseases (Appay et al., 2002). In the case of LCMV infection in mice, killing was shown to be MHC-class II dependent and antigen-specific (Jellison et al., 2005; Nikiforow et al., 2003).

Cytotoxic $CD4^+$ T cells may function through a variety of mechanisms, including ligation of the death receptor Fas on target cells with its ligand (FasL) on $CD4^+$ T cells, and production of IFN-γ and TNF-α (Stalder et al., 1994). Alternatively they have been shown to act through production of perforin and granzymes, in a mechanism similar to that of cytotoxic $CD8^+$ T cells (Williams and Engelhard, 1996).

1.3.4 Memory $CD4^+$ T cells

As described above, immunological memory is defined as the ability of a host to recognise a secondary challenge by a pathogen, and mount a more sensitive and effective response in order to prevent establishment of infection. After expansion of naive $CD4^+$ T cells in response to antigen, a contraction of cell numbers takes place with ~90% of antigen-specific $CD4^+$ T cells undergoing programmed cell death, and the remaining 10% enter the memory $CD4^+$ T cell pool. Memory $CD4^+$ T cells are constantly replenished by low level differentiation, at a higher rate than that of naive $CD4^+$ T cells (Macallan et al., 2004). A combination of cytokines, T cell and APC interactions, and balanced expression of anti-apoptotic molecules is necessary for successful generation, and efficient maintenance of memory $CD4^+$ T cells (Lanzavecchia and Sallusto, 2002).

Memory $CD4^+$ T cells can be further divided into effector memory (T_{EM}) or central memory (T_{CM}) cell subsets, depending on their level of expression of CCR7 and CD62L (L-selectin). CCR7 is a chemokine receptor which directs migration of cells to secondary lymphoid organs, while CD62L is an adhesion molecule which binds to ligands on epithelial cells, also controlling migration (Butcher and Picker, 1996; Sallusto et al., 1999). T_{EM} $CD4^+$ cells are $CCR7^{low}CD62L^{low}$ and are the first memory $CD4^+$ T cells to respond to an assault by a pathogen, expressing tissue-homing β1 and β2 integrins and inflammatory chemokine receptors, which enable them to migrate to inflamed tissues and mount an effector $CD4^+$ T cell response. T_{CM} $CD4^+$ cells are $CCR7^{high}CD62L^{high}$, and reside in the lymph nodes where they remain until they are prompted to differentiate into effector cells and migrate to the site of the secondary infection (Sallusto et al., 1999).

The life span of memory $CD4^+$ T cells is not currently well defined. In murine studies LCMV-specific memory $CD8^+$ T cells can be stably maintained throughout the lifetime of the host. However, LCMV-specific memory $CD4^+$ T cell number, but not function, declines over time, albeit with a slower initial contraction phase than that of the LCMV-specific $CD8^+$ T cell population (Homann et al., 2001). Memory $CD4^+$ T cells have also been shown to decline in *Listeria monocytogenes* infection, while bacteria-specific $CD8^+$ memory T cells were able to be maintained (Schiemann et al., 2003). In contrast it was also shown that despite induction of memory $CD8^+$ T cells during acute infection, memory $CD8^+$ T cell effector function declined over time during LCMV infection (Fuller et al., 2004).

In human studies on patients vaccinated against smallpox, vaccinia-specific memory $CD4^+$ T cells were present more than 60 years after subjects had been immunised. Furthermore, these $CD4^+$ T cells had retained effector function (Hammarlund et al., 2003). Chimpanzees vaccinated with an HIV-1 vaccine candidate maintained antigen-specific $CD4^+$ T cells for five years post-immunisation while the population of antigen-specific $CD8^+$ T cells declined. This was further observed in 1 of 4 human volunteers (Balla-Jhagjhoorsingh et al., 2004).

1.3.5 Homeostatic cytokines

Newly developed memory $CD4^+$ T cells must compete with original memory cells for access to homeostatic cytokines which are most often from the common γ chain (γc) cytokine family, and in order to receive signals from these cytokines cells must express the relevant receptor on their cell surface.

IL-7 plays an important role in the generation of memory $CD4^+$ T cells, as well as enhancing survival of effector $CD4^+$ T cells. IL-7Rα is downregulated on activated T cells and re-expressed at high levels on memory T cells. IL-7 has been shown to be essential for the generation of memory $CD4^+$ T cells from adoptively transferred effector $CD4^+$ T cells in IL-7-deficient mice, and for generation of memory $CD4^+$ T cells from effector $CD4^+$ T cells in WT mice treated with an IL-7-blocking antibody (Li et al., 2003). Furthermore, IL-7 was shown to be necessary for survival of memory $CD4^+$ T cells via the upregulation of the anti-apoptotic molecule Bcl-2, with memory cells unable to persist in IL-7-deficient mice, or in mice treated with an IL-7 blocking antibody (Kondrack et al., 2003). Concerted IL-7 and TCR signalling was shown to be necessary for optimum maintenance of memory $CD4^+$ T cells. However, deficiency of IL-7 or abrogation of TCR signalling can be overcome by an increase of the other and hence display an element of redundancy (Seddon et al., 2003).

As well as inducing T cell proliferation, IL-2 contributes towards blocking cell death via induction of anti-apoptotic molecules such as Bcl-2. However, it also inhibits cycling of $CD4^+$ T memory cells, and although IL-2Rα is up-regulated on effector

$CD4^+$ T cells, it is expressed at low levels on memory $CD4^+$ T cells (Marrack et al., 2000).

IL-15 is produced by activated macrophages and DCs in response to infection and its role in the maintenance of memory T cells has mostly been studied in memory $CD8^+$ T cells. IL-15R deficient mice have normal numbers of memory $CD4^+$ T cells but it is possible that compensatory mechanisms are in place, similar to those demonstrated in the absence of IL-7 (Seddon et al., 2003). These and other observations lead to the conclusion that IL-15 was required for $CD8^+$ T cell homeostasis but not for $CD4^+$ T cell homeostasis (Tan et al., 2002). However, more recently it has been shown that anti-viral memory $CD4^+$ T cells require IL-15 for homeostatic survival, to the same extent as memory $CD8^+$ T cells (Purton et al., 2007).

Relative expression of homeostatic cytokine receptors on T cells is shown in **Figure 1.1**.

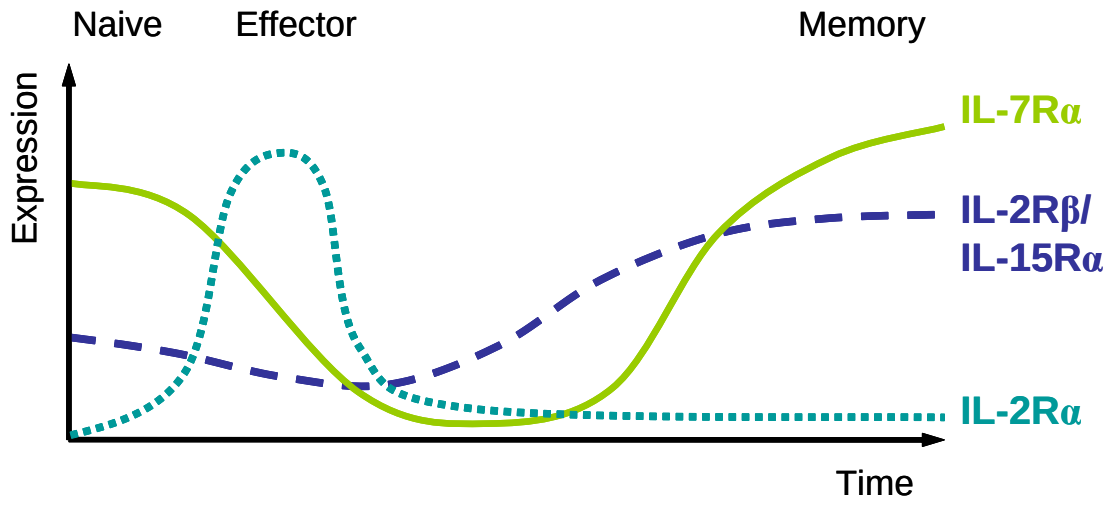

Figure 1.1 Expression of homeostatic cytokine receptors at different stages of the CD4$^+$ T cell life cycle

CD4$^+$ T cells express receptors in order to receive survival signals from the homeostatic γc cytokines IL-2, IL-15 and IL-7. Receptors for these cytokines are expressed at different levels at different stages of CD4$^+$ T cell differentiation from a naive phenotype through to effector and memory cells. Adapted from (Schluns and Lefrancois, 2003).

1.3.6 Progressive differentiation and T cell exhaustion

A model of progressive differentiation has been described whereby T cells differentiate to a certain level depending on the amount of antigen stimulation received. Each stage of differentiation is characterised by a different cytokine profile and cells can enter the memory pool at any of these stages (Lanzavecchia and Sallusto, 2000). This model of progressive differentiation can also be applied to the memory $CD4^+$ T cell population (Seder et al., 2008).

In the case of Th1 cells, in the early phases of differentiation from naive to effector $CD4^+$ T cells they produce only IL-2. Increased antigen stimulation switches on production of IFN-γ, turning cells into double-producers, while higher levels of antigen stimulation leads to cessation of IL-2 production and hence a IFN-γ single-producer population (**Figure 1.2**). Cells which receive only weak stimulation do not survive, while at very high levels of activation they ultimately become exhausted and succumb to deletion from the immune cell pool (Lanzavecchia and Sallusto, 2005).

T cell exhaustion and deletion can occur during chronic viral infection when antigen persists at high levels, as demonstrated in $CD8^+$ T cells in LCMV infection (Moskophidis et al., 1993). This exhaustion of virus-specific T cells has also been shown in chronic viral infections in humans, including EBV, CMV, hepatitis B and C, and HIV (Klenerman and Hill, 2005). As well as reduced frequency due to deletion after chronic activation, features of T cell exhaustion include reduced functionality of antigen-specific T cells, downregulation of co-stimulatory molecules such as CD127 and CD62L, and increased expression of T cell inhibitory molecules such as PD-1 (Yi

et al., 2010). Notably, during T cell exhaustion during LCMV infection in mice, blocking interactions of PD-1 with its ligand (PD-1L) allowed recovery of antigen-specific $CD8^+$ T cell function and number (Barber et al., 2006).

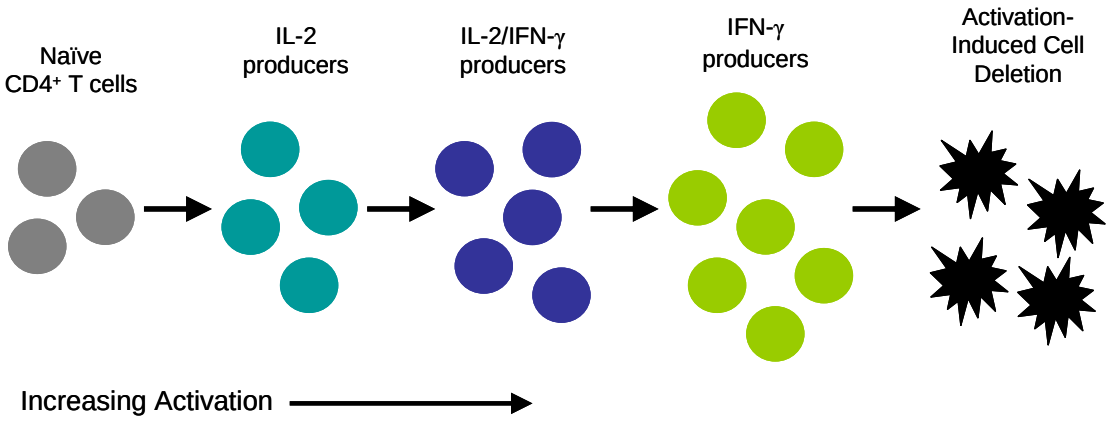

Figure 1.2 Progressive differentiation of Th1 CD4$^+$ T helper cells

Naive T cells migrate from the thymus to the periphery where they encounter antigen and differentiate into effector T cells, producing different cytokines depending on the level of antigen stimulation received. In this example, Th1 CD4$^+$ T cells produce IL-2 at low levels of antigen stimulation, go on to become IL-2 and IFN-γ double-producers at higher levels of antigen stimulation. They then cease producing IL-2 to become IFN-γ single-producers before undergoing activation-induced cell-deletion at the highest levels of antigen stimulation. Adapted from (Lanzavecchia and Sallusto, 2005).

1.4 Retroviruses

Retroviruses are enveloped, single stranded RNA viruses which can be divided into five groups of simple retroviruses, which are oncogenic, and two groups of complex retroviruses – lentiviruses and spumaviruses. Oncogenic retroviruses include human T cell leukaemia virus (HTLV) and murine leukaemia virus (MLV), while HIV is a member of the lentiviridiae family.

1.4.1 Retroviral structure

Retroviral virions have a lipid bilayer envelope studded with envelope glycoproteins. The retroviral genome, which contains single stranded RNA and viral enzymes, is encased in a protein capsid (**Figure 1.3A**). All retroviruses have three key protein coding genes: *gag*, *env* and *pol*. *Gag* encodes for virion proteins, *env* for viral envelope proteins and *pol* for reverse transcriptase, integrase and protease - the enzymes involved in viral replication (**Figure 1.3B**). Complex retroviruses have additional genes in their genome such as *rev*, *tat*, *nef*, *vpu*, *vif* and *vpr*, encoding for early proteins which have roles such as promoting immune evasion, and capsid uncoating (Nisole and Saib, 2004).

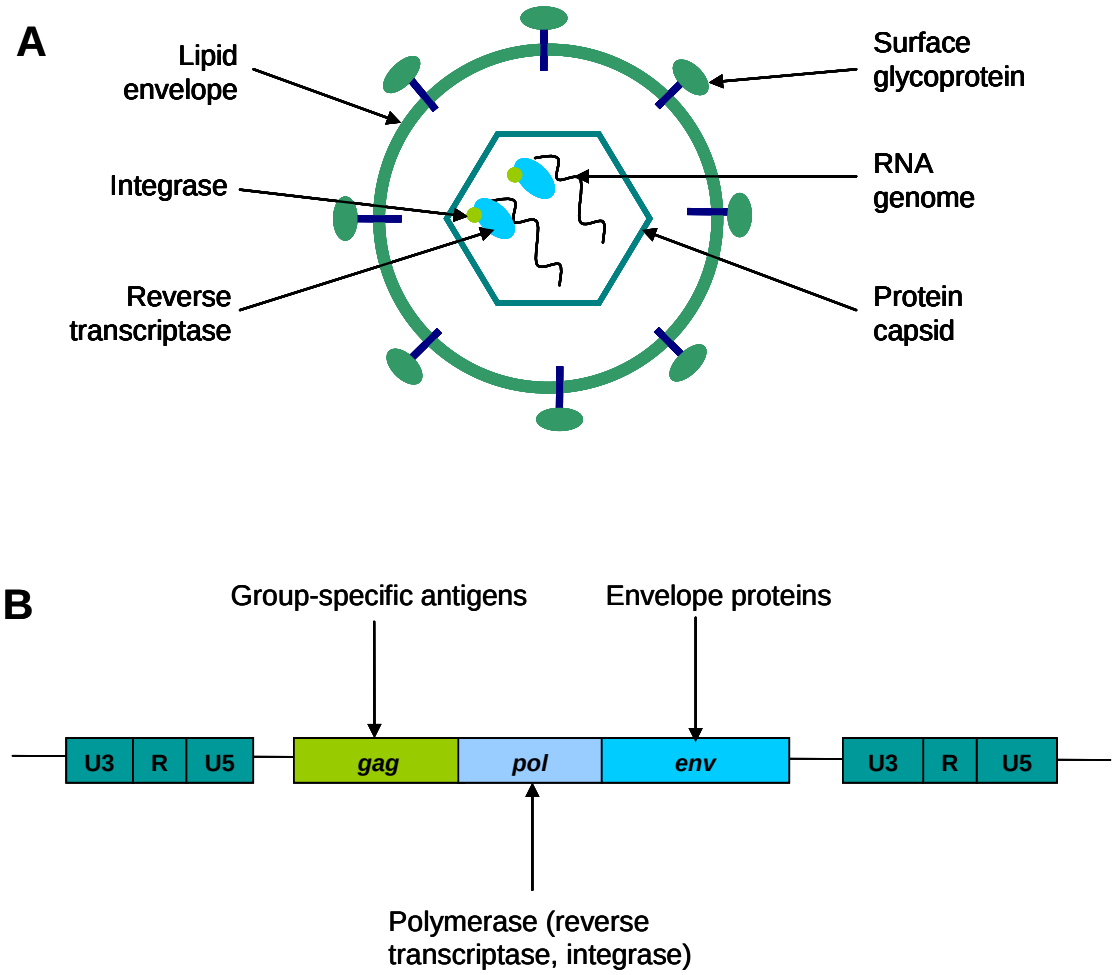

Figure 1.3 The generic structure and RNA genome of a simple retrovirus

(A) The RNA genome of a retrovirus is contained within a protein capsid. This is surrounded by a lipid envelope which is studded with glycoproteins. These surface glycoproteins allow the virion to attach to its target cell membrane via receptors and gain entry into the cell.

(B) The RNA genome of a simple retrovirus contains 3 major genes: gag which encodes for surface antigens, pol which encodes for enzymes required for retroviral replication, and env which encodes for envelope proteins in the virion.

1.4.2 The retroviral life cycle

Retroviral envelope glycoproteins interact with receptors on the surface membrane of the host target cell in order to gain entry. In HIV for example, the glycoprotein gp120 on the virus interacts with CD4 and CCR5 or CXCR4 molecules and a virological synapse forms. There are two ways in which a retrovirus can enter its target cell during infection: fusion of the viral envelope, as observed in HIV, or via endocytosis, as observed in murine leukaemia viruses (MLVs) (Katen et al., 2001). After cell entry, the viral protein capsid is released into the cell and reverse transcription of viral RNA to viral cDNA mediated by the viral reverse transcriptase enzyme occurs. Viral cDNA subsequently enters the host cell nucleus and integrates into the host cell genome. While lentiviruses such as HIV are able to integrate into the genome of non-replicating cells, potentially by entering the nucleus via a nuclear pore, MLVs require cell division to take place in order for them to integrate (Nisole and Saib, 2004). Integration into the host genome is not random and favours active genes (Schroder et al., 2002; Wu et al., 2003).

Viral DNA is then transcribed in viral RNA, viral genes are expressed and new virion components are produced in the cytoplasm. These components assemble to form a new virus particle which traffics to the cell membrane via interactions within the host cytoskeleton (Naghavi and Goff, 2007). The virion then uses the host cellular machinery to exit the cell, budding from the cell surface (Perez and Nolan, 2001). Once the retroviral virion has budded from the cell it matures and the cycle begins again (Naghavi and Goff, 2007).

1.5 Human Immunodeficiency Virus

1.5.1 HIV/AIDS

Despite only first being described several decades ago (Barre-Sinoussi et al., 1983), HIV/AIDS represents a major global health problem with more than 33 million people infected worldwide, and an estimated 2.5 million new infections occurring during 2009 (www.who.int). Infection with HIV begins with an acute phase characterised by high viral load and flu-like symptoms, and a subsequent chronic phase where viral load persists at a lower level asymptomatically. Ultimately, profound loss of function and depletion of $CD4^+$ T cells in the host leads to immunocompromisation and susceptibility to fatal opportunistic infections such as *Pneumocystis jiroveci* and cancers such as Kaposi's sarcoma. This stage of disease is known as acquired immune deficiency syndrome (AIDS).

During peak HIV viraemia, the major sites of $CD4^+$ T cell depletion are mucosal tissues and associated lymphoid tissues, with up to 80% of $CD4^+$ T cells depleted from the gut-associated lymphoid tissue during initial infection (McMichael et al., 2010; Brenchley and Douek, 2008). There is evidence showing that although $CD4^+$ T cell depletion occurs at a range of sites, the gastro-intestinal tract remains the principal site of this depletion throughout HIV infection (Brenchley et al., 2004). Several mechanisms underlying the loss of $CD4^+$ T cells during HIV infection have been described. The major mechanism has not yet been determined, and it is likely that several mechanisms contribute to depletion of $CD4^+$ T cells during HIV infection (McCune, 2001a).

As well as cytopathic infection of $CD4^+$ T cells by the virus and killing of infected cells by cytotoxic $CD8^+$ T cells, immune activation has been shown to play a key role in destruction of the $CD4^+$ T cell pool during both the acute and chronic phases of infection (McMichael et al., 2010). Chronic activation of $CD4^+$ T cells during HIV infection results in overactivation of these cells, resulting in them becoming hyperactivated and exhausted, and subsequently undergoing activation-induced cell death (McCune, 2001b). Activated $CD4^+$ T cells are also more susceptible to infection by HIV, and increased numbers of these cells will therefore promote spread of the virus (McMichael et al., 2010). B cells and $CD8^+$ T cells can also be damaged due to chronic immune activation during prolonged HIV infection, resulting in further reduction of overall immune function (McMichael et al., 2010).

A small proportion of HIV-infected individuals are able to control progression to AIDS in the absence of antri-retroviral drugs. These include long-term non-progressors (LTNPs) and elite controllers. LTNPs are able to retain a normal $CD4^+$ T cell count despite infection with HIV and high viral load, while elite controllers are able to control viral load, maintaining it at a low level and hence controlling progression to AIDS (Blankson, 2010). These populations provide opportunities to investigate mechanisms which lead to control of HIV infection, prevention of $CD4^+$ T cell loss and delay of AIDS onset, and may provide valuable clues for vaccine development.

Despite the development and increased distribution of highly active anti-retroviral therapy (HAART) as a treatment for HIV infection, a vaccine is the only way in

which to effectively prevent the spread of HIV and control this global epidemic. However, development of a vaccine to protect against infection by HIV remains a seemingly unachievable task. Since its discovery in 1985, numerous candidate vaccines have been proposed and investigated to no avail. Some recent examples of clinical trials for prophylactic HIV vaccines are described below.

1.5.2 Development of an HIV vaccine

In the Merck Step Study a replication-incompetent adenovirus serotype 5 (Ad5) vector vaccine containing *gag*, *pol* and *nef* genes was trialled, but phase II trials showed that the vaccine was not able to provide protection against HIV infection. In fact, there was a trend towards an increased susceptibility to HIV infection in those participants which had previous adenoseropositivity upon commencing the trial. This observation resulted in premature termination of the trial (Buchbinder et al., 2008). Further analysis revealed that the vaccine induced functional HIV-specific $CD4^+$ and $CD8^+$ T cell responses regardless of initial Ad5 antibody titre, but was not able to reduce viral load in those who became infected after receiving the vaccine (McElrath et al., 2008). Further study showed that *in vitro* stimulation of T cells from healthy volunteers who were adenovirus seropositive resulted in expansion of adenovirus specific memory $CD4^+$ T cells. These $CD4^+$ T cells had a mucosal homing phenotype and were particularly susceptible to infection by HIV, potentially explaining why those volunteers who were adenoseropositive at the beginning of the trial were more susceptible to infection by HIV (Benlahrech et al., 2009).

A trial carried out in Thailand, where volunteers were vaccinated with a canarypox vector vaccine and subsequently boosted with a recombinant gp120 vaccine, showed a potentially promising outcome. Preliminary studies showed that $CD8^+$ T cell responses and nAb responses were induced by the vaccine regimen (Nitayaphan et al., 2004). The phase III clinical trial showed that the vaccine was adequate to protect against HIV infection with 30% efficacy. However, no reduction of viral load or preservation of $CD4^+$ T cells was seen in those patients who did contract the virus after vaccination compared to the placebo group (Rerks-Ngarm et al., 2009). Although protection was low, this study provides a promising basis for further examination. These clinical trials show that much more study is required into the specific immune responses required to protect against HIV, as well as the vaccination approaches necessary to induce such an immune response.

1.5.3 Obstacles to vaccinating against HIV

The major obstacle in the development of an effective vaccine to prevent HIV infection is the high level of antigenic variability of retroviruses, enabling HIV to successfully evade recognition by the immune response. As well as the antigen variability observed between strains, reverse transcriptase, the viral enzyme involved in reverse transcription, has a highly error-prone nature (Ji and Loeb, 1992; Roberts et al., 1988). This high error-rate of retroviral reverse transcriptase is due in part to its lack of exoribonuclease proof reading mechanism (Svarovskaia et al., 2003). Another factor contributing to the variability of HIV is the high rate of HIV replication. The virus has a relatively short half-life of approximately two days, while mathematical models have estimated that 10.3×10^9 HIV virions are produced per day (Perelson et al., 1996; Wei et al., 1995). Together, this results in constantly altering epitopes, and hence makes selecting appropriate epitopes for target with vaccines a complex challenge.

1.5.4 Role of $CD4^+$ T cells against HIV infection

HIV-specific $CD4^+$ T cells are present in HIV infected individuals, and a strong HIV-specific $CD4^+$ T cell response is generally associated with slow rate of disease progression (Lichterfeld et al., 2005). However, as HIV also preferentially infects HIV-specific $CD4^+$ T cells (Douek et al., 2002), it is unclear whether a strong virus-specific $CD4^+$ T cell response is the cause or simply the consequence of low viral replication.

Patients who are able to control viraemia without HAART show vigorous HIV-specific $CD4^+$ T cell responses, and the magnitude of these responses correlates inversely with viral load, while acutely infected individuals treated with HAART develop strong $CD4^+$ T cell responses, again correlating with a reduced viral load (Rosenberg et al., 1997). However, it has also been shown that HAART-mediated prolonged suppression of HIV viraemia during infection can leads to loss of HIV-specific $CD4^+$ T cells (Pitcher et al., 1999).

As mentioned with regards to other viral infections, cytotoxic $CD4^+$ T cells have also been identified in HIV. HIV Gag-specific $CD4^+$ T cell clones derived from infected individuals have been shown to have perforin-dependent cytolytic ability *in vitro* as well as producing IFN-γ (Norris et al., 2001). This cytotoxic function of HIV-specific $CD4^+$ T cells against HIV-infected cells was further demonstrated *ex vivo*, and direct inhibition of replication in a cell contact-dependent but IFN-γ-independent manner was observed (Norris et al., 2004).

SIV Gag- and Nef-specific $CD4^+$ T cells were shown to be able to directly inhibit replication of HIV in infected macrophages but not in other infected $CD4^+$ T cells (Sacha et al., 2009), while HIV Nef-specific $CD4^+$ T cells were also shown to inhibit viral replication in both macrophages and $CD4^+$ T cells (Zheng et al., 2009).

Although the correlation between control of viral load and a strong HIV-specific $CD4^+$ T cell response has been demonstrated extensively, the cause and effect relationship between the $CD4^+$ T cell response and its role in controlling HIV infection, as well as the major mechanisms by which $CD4^+$ T cells control HIV infection, remain undetermined.

1.5.5 Role of $CD8^+$ T cells against HIV infection

The resolution of the acute phase of HIV infection and control of viral load is primarily attributed to $CD8^+$ T cells (McMichael et al., 2010). Using SIV infection as an animal model for HIV infection, it was shown that depleting $CD8^+$ T cells during acute infection rendered animals unable to control viral replication, while depleting $CD8^+$ T cells in chronically infected animals resulted in increased viremia, which was resolved upon restoration of the $CD8^+$ T cell pool (Schmitz et al., 1999).

$CD8^+$ T cells are also shown to be at higher frequencies in LTNPs, possibly explaining the ability of this population of HIV-infected persons to delay the onset of AIDS. These $CD8^+$ T cells were shown to be able to kill HIV-infected $CD4^+$ T cells via administration of granzyme B, more efficiently than those $CD8^+$ T cells from progressors (Migueles et al., 2008). These findings demonstrate the importance of

$CD8^+$ T cells in HIV infection, and in particular the importance of their cytolytic capacity.

Both avidity and polyfunctionality of $CD8^+$ T cells have been shown to be correlative with effective control of HIV replication (Almeida et al., 2007). Furthermore, HIV-specific $CD8^+$ T cells of high avidity have been shown to have increased polyfunctional ability compared to those of low avidity (Almeida et al., 2009). $CD8^+$ T cells specific for HIV have also been shown to inhibit replication of the virus in macrophages more than they can in $CD4^+$ T cells (Fujiwara and Takiguchi, 2007). This difference is due to downregulation of MHC class I by Nef on $CD4^+$ T cells, occurrence of this on macrophages is negligible.

A recently identified role for $CD4^+$ T cell-derived IL-21 in viral infection was discussed above. In HIV, it was found that IL-21-producing $CD4^+$ T cells were present during acute and chronic HIV infection, and that elevated levels of these cells were associated with control of viral load via maintenance of $CD8^+$ T cells (Yue et al., 2010). A further study confirmed the requirement for these IL-21 producing $CD4^+$ T cells in control of HIV infection, showing IL-21 mediated enhancement of $CD8^+$ T cell cytotoxic activity (Chevalier et al., 2010).

A major problem limiting the effectiveness of $CD8^+$ T cells in controlling HIV infection is immune escape by the virus (McMichael et al., 2010; Davenport et al., 2008). During acute SIV infection, $CD8^+$ T cell-resistant escape variants of SIV were shown to develop (O'Connor et al., 2002). Furthermore, during acute HIV infection

virus mutations developed in $CD8^+$ T cell epitopes (Bernardin et al., 2005). This immune escape has hindered the development and limited efficacy of HIV vaccines designed to induce $CD8^+$ T cells against the virus.

1.5.6 Inducing $CD4^+$ T cells against HIV

Although the above studies show that $CD4^+$ T cells clearly play a role in controlling HIV infection, inducing them can also have a detrimental effect. HIV has been shown to preferentially infect HIV-specific $CD4^+$ T cells, suggesting that inducing HIV-specific $CD4^+$ T cells may not only be ineffective but could actually be harmful to the host (Douek et al., 2002). Furthermore, vaccinations that elicit a $CD4^+$ T cell response have been shown to have a damaging effect in both animal and human subjects. In HIV-infected individuals, vaccination with influenza vaccine resulted in a boost in viral replication and increased HIV plasma viral load, correlating with induction of influenza-specific T cell responses (Staprans et al., 1995). Other studies vaccinating with the *env* gene, as a DNA vaccine in cats against Feline Immunodeficiency Virus (FIV) has shown, result in advancement of the acute phase of infection (Richardson et al., 1997). Furthermore vaccination of macaques against SIV induced $CD4^+$ T cells but also resulted in increased viral replication and a more rapid onset of simian AIDS (Staprans et al., 2004).

Other vaccinations where $CD4^+$ T cells were induced have been shown to be protective against infection. Vaccination against SIV Gag using a lentiviral vector was also able to protect against SIV infection, again correlating with preservation of SIV-specific memory $CD4^+$ T cells during the acute phase of infection (Beignon et al.,

2009). Additionally, transfer of activated $CD4^+$ T cells resulted in expansion of endogenous T cells, immune reconstitution, increased $CD4^+$ T cell count and reduced expression of CCR5 (Bernstein et al., 2004; Levine et al., 2002).

In conclusion, these latter studies indicate that $CD4^+$ T cells are an essential component in immune protection against HIV. Indeed, it has been shown that $CD4^+$ T cell epitopes on HIV-1 may be subject to less variation than $CD8^+$ T cell epitopes (Koeppe et al., 2006). This suggests that $CD4^+$ T cells would provide a less variable target for vaccination, and that employing a vaccine strategy that induces $CD4^+$ T cells could be more widely efficient and resist the variation seen in HIV epitopes. Hence, the contribution of $CD4^+$ T cells to control of HIV infection is not clearly defined and requires further investigation.

1.6 Friend Virus

1.6.1 The Friend virus complex

Friend Virus (FV) provides a useful and adaptable murine model in which the immune response to retroviral infection can be investigated. FV was discovered after the observation that leukaemia could be transferred into adult mice by inoculating them with a cell-free filtrate from spleens of leukemic mice. Infection of susceptible mice with this virus results in two stages of Friend disease: splenic enlargement and subsequently, fatal erythroleukaemia (Friend, 1957).

The FV retroviral complex is comprised of replication-competent Friend Murine Leukaemia helper virus (F-MuLV) and replication-defective spleen focus-forming virus (SFFV). The SFFV *env* gene encodes gp55 - an envelope fusion glycoprotein which mimics erythropoietin (Epo) and binds to and causes the constitutive activation of the erythropoietin receptor (EpoR). Ligation of gp55 with the EpoR on the cell-surface and endoplasmic reticulum of FV-infected cells causes expansion of BFU-E (burst-forming units-erythroid) and CFU-E (colony-forming units-erythroid), and results in prolonged polyclonal proliferation of erythroid precursor cells, leading to splenic enlargement and erythroleukaemia (Li et al., 1990; Ney and D'Andrea, 2000). FV infection persists in the spleen of mice after recovery and its primary reservoir has been identified as a small population of B cells, rather than erythroid precursors which comprise the majority of the infected cell population during acute infection (Chesebro et al., 1979; Hasenkrug et al., 1998).

Although the FV complex does not contain an oncogene, mutations are acquired as mice progress into erythroleukaemia. Pro-viral integration of the SFFV component of the FV complex occurs at the host gene Spi-1 (SFFV proviral integration 1, also known as PU.1) locus, an oncogene which is only seen in murine erythroleukaemia and not myeloid or lymphoid leukaemia (Moreau-Gachelin et al., 1988). During FV infection, rearrangement of, and mutations in, the p53 gene lead to truncation of the protein or complete abrogation of protein expression. Both result in p53 inactivation, a process which is necessary for development of Friend erythroleukaemia (Munroe et al., 1990). Overall, FV-induced erythroleukaemia is characterised by activation of EpoR and Spi-1, and inactivation of p53.

Entry of murine leukaemia viruses, including FV, into rodent cells occurs via a membrane receptor designated as the ecotropic MuLV receptor. This receptor is an amino acid transporter, specifically transporting cationic amino acids across the cell membrane (the cationic amino acid transporter, or CAT). This receptor is ubiquitously expressed in mice, but notably is not expressed in hepatocytes, commensurate with the observation that murine leukaemia viruses do not infect cells in the liver (Kim et al., 1991; Wang et al., 1991). Additionally, binding of the viral gp70 envelope glycoprotein to this receptor prevents further infection of the cell via this receptor, but not amino acid transportation (Kim et al., 1991; Overbaugh et al., 2001). It was thought that CAT-1, which is not expressed in the liver was the receptor for ecotropic MuLV, while CAT-2 which is expressed in the liver, was the receptor for amphotropic MuLV. However, while CAT-1 expression is essential for infection of hepatocytes by ecotropic virus, neither CAT-2A or CAT-2B isotypes are adequate to allow infection of hepatocytes by amphotropic virus (Closs et al., 1993).

1.6.2 Susceptibility to FV-induced pathology

Susceptibility to, and recovery from, FV infection differs between mouse strains and can be determined by both immunological and non-immunological host genes (Chesebro et al., 1974). Differences in the MHC *H-2* haplotype control susceptibility to splenomegaly, erythroleukaemia and immunosuppression, (Morrison et al., 1987; Morrison et al., 1986). These are summarised in **Table 1.1**. B6 mice are *H-2*$^{b/b}$, while C mice are *H-2*$^{d/d}$ and hence are fully susceptible (Hasenkrug and Chesebro, 1997).

The high incidence recovery *H-2*$^{b/b}$ haplotype is within the *H-2D* region and is thought to exert its effect via regulation of virus-specific cytotoxic CD8$^+$ T cells. Other loci which contain genes affecting the immune response to FV include the H-*2A, E* and *Qa/Tia* regions. Expression of *H-2E* has been shown to have both positive and negative effects on recovery from FV (Perry et al., 1994).

The *H-2D* MHC haplotype can also affect the number of IFN-γ producing CD8$^+$ and CD4$^+$ T cells. Thus, *H-2*$^{b/b}$ mice have high numbers of IFN-γ producing cells and *H-2*$^{a/b}$ and *H-2*$^{d/d}$ mice have lower numbers, correlating with the differences in recovery seen in mice of this genotype (Peterson et al., 2000). It has also been shown that MHC class I is able to modify the CD4$^+$ T cell response to FV. This requires the presence of CD8$^+$ T cells and so suggests an unconventional provision of help to CD4$^+$ T cells from CD8$^+$ T cells (Peterson et al., 2000; Peterson et al., 2002).

MHC Genotype	Effect of FV Infection
H-2$^{a/a}$	Splenomegaly and fatal erythroleukaemia
H-2$^{b/b}$	Initial splenomegaly followed by spontaneous recovery
H-2$^{d/d}$	Fully susceptible to splenomegaly and erythroleukaemia
H-2$^{a/b}$	Low dose infection - splenomegaly then recovery No recovery from high dose infection
H-2$^{b/d}$	Can not recover from splenomegaly and die

Table 1.1 Summary of susceptibility to FV infection

The *Rfv-1* and *Rfv-2* genes are both MHC-linked and affect recovery from FV disease (Chesebro and Wehrly, 1978; Miyazawa et al., 1995). In contrast, *Rfv3* is a non-MHC gene which controls induction of FV-specific antibodies and recovery in association with *H-2* genes and has no effect on susceptibility to immunosuppression (Morrison et al., 1986).

Compared to *H-2*$^{a/b}$ or *H-2*$^{b/b}$ *Rfv-3*$^{r/s}$ mice, animals with an *H-2*$^{a/a}$ *Rfv-3*$^{r/s}$ genotype cannot mount an FV-specific T cell response. Although they can mount a virus-neutralising IgM antibody response, they cannot class-switch to IgG and thus have a low recovery phenotype. However, induction of the nAb response in all three *H-2* genotype *Rfv-3*$^{r/s}$ mice is dependent on the presence of CD4^{+} T cells, and viraemia is increased in their absence. Furthermore, in the absence of CD4^{+} T cells, *H-2*$^{a/a}$ *Rfv-3*$^{r/s}$ mice do not recover from FV-induced splenomegaly, showing a requirement for CD4^{+} T cell help in FV infection of *Rfv-3*$^{r/s}$ mice independent of *H-2* genotype. Proliferation of CD4^{+} T cells has been seen after FV infection in *H-2*$^{a/a}$ *Rfv-3*$^{r/s}$ mice but their specificity was not known (Super et al., 1998).

1.6.3 Friend virus susceptibility genes

In addition to MHC variability, FV disease pathogenesis and susceptibility to disease in mice is controlled by a broad range of non-MHC host genes – *Friend virus susceptibility genes 1-6* (*Fv1-Fv6),* which are able to confer their effects via both immunological and non-immunological mechanisms (Bieniasz, 2003).

The *Fv1* locus was the first restriction factor to be described. Restriction factors are also found in humans (e.g. *Restriction factor 1* (*Ref1)* in HIV) and have developed throughout evolution to prevent integration of retroviruses into the host genome (Goff, 2004). The *Fv1* gene encodes Fv1, which is derived from an endogenous retrovirus and restricts establishment of the viral RNA genome after it has entered the cell and reverse transcription has occurred (Best et al., 1996). Fv1 is related to an endogenous retrovirus Gag-related protein, and in laboratory mouse strains two major alleles exist: $Fv1^n$ and $Fv1^b$ (from NIH and BALB/c mice respectively). Depending on their ability to replicate in cells carrying either the $Fv1^n$ or $Fv1^b$ allele, different strains of MuLV are allocated as N-tropic, B-tropic or NB-tropic. The $Fv1^n$ allele restricts B-tropic virus while the $Fv1^b$ allele restricts N-tropic virus. NB-tropic virus strains can replicate in cells with either or both alleles, while cells that are heterozygous are resistant to both N- and B-tropic strains. Furthermore, Fv1 is saturable, and its restriction can be overridden by increased viral load (Bieniasz, 2003; Pryciak and Varmus, 1992; Bieniasz, 2004).

The *Fv2* gene controls susceptibility to FV-induced splenomegaly and erythroleukaemia by determining gp55-mediated proliferation of SFFV-infected

erythroblasts. In *Fv2* susceptible (*Fv2*^{s}) mice the *Fv2* gene encodes for both a full length and truncated form of a receptor tyrosine kinase, known as Stk and sf-Stk respectively (Persons et al., 1999). However, in *Fv2* resistant (*Fv2*^{r}) mice the *Fv2* gene encodes only for Stk, and as sf-Stk is required for FV-mediated erythroproliferation, these mice are resistant to splenomegaly (Finkelstein et al., 2002). Sf-Stk lacks the N-terminal ligand binding domain and confers susceptibility of erythroid cells to proliferation via covalent interactions between cysteine residues in its extracellular domain and in the gp55 molecule, resulting in constitutive activation of the EpoR (He et al., 2010). While most laboratory mouse strains are *Fv2*^{s}, C57BL/6 (B6) mice and related strains are *Fv2*-resistant (*Fv2*^{r}) (Persons et al., 1999; Ney and D'Andrea, 2000). However, B6 mice with susceptibility at the *Fv2* allele (B6.A-*Fv2*^{s}) are susceptible to FV-induced splenomegaly compared to wild-type B6 mice, although they recover from this splenomegaly. In contrast, B6 mice with susceptibility at the *Fv2* allele and a deficiency in adaptive immune cells (B6.A-*Fv2*^{s}*Rag1*$^{-/-}$ mice) suffer acute splenomegaly which is fatal (Marques et al., 2008).

The *Fv4* gene blocks cell-surface receptors by expressing an endogenous retroviral envelope protein that competes with F-MuLV env for ligation of these receptors (Ikeda et al., 1985). Whilst *Fv3* and *Fv5* have not been clearly established as independent loci, *Fv3* is known to regulate FV-induced immunosuppression, potentially by regulating the number or function of Tregs (Morrison et al., 1986), while *Fv6* determines resistance to early onset erythroleukaemia (Shibuya and Mak, 1982).

1.6.4 FV infection as a mouse model of HIV

There are several problems associated with studying the immune response to HIV. Immune responses in *ex vivo* samples from HIV-infected patients can be studied but the exact duration and stage of infection are often difficult to determine, and so parameters of the immune response during different phases of infection cannot be examined. Additionally, human research trials require ethical approval. Non-human primates provide a laboratory animal model in which the homologous SIV is used, but these have time and cost limitations as well as ethical considerations.

HIV host range does not extend to rodents and this is unfortunate because gene knockout mice and transgenic mouse models provide powerful tools for investigating the role of individual cell types and molecules such as cytokines and MHC, and to study pathogen-specific immune responses. Additionally, in mouse models the immune response can be monitored and studied in detail at defined points during infection.

The major pathogens used in mouse models to study the immune response to viral infection include and vesicular stomatitis virus (VSV), and while these have provided valuable information regarding anti-viral T cell responses, the significance for understanding the immune response to HIV is limited as they are not retroviruses. A murine model which has been widely utilised to study the immune response to chronic viral infection is infection with lymphocytic choriomeningitis virus (LCMV). In particular, LCMV Clone 13 which results in chronic infection and T cell exhaustion has been useful, as opposed to LCMV Armstrong which causes an acute

and relatively rapidly cleared infection. Several observations concerning the immune response during LCMV Clone 13 infection are in line with those described in HIV infection with regards to $CD4^+$, $CD8^+$ and B cell responses, as well as data concerning T cell exhaustion. However, while parallels to HIV can be drawn from results from studies in LCMV, LCMV is not a retrovirus, and hence FV provides an alternative model for studying immune responses to chronic retroviral infection.

In contrast to these models, the retroviral complex FV exhibits many analogous properties to HIV, including similar mechanisms of replication via the error prone enzyme reverse transcriptase, and integration into the host cell genome (Nisole and Saib, 2004). Furthermore, both viruses are able to replicate in macrophages and have many common targets of infection. Although the major target of FV is erythroid precursors which FV infects via CAT-1, while the major target of HIV is $CD4^+$ T cells which HIV infects via CD4 and either CCR5 or CXCR4, these differences allow us to dissociate from the direct effect of viral infection on the $CD4^+$ T cell response to HIV from those of antigen presentation to $CD4^+$ T cells in FV. Additionally, FV and HIV both persist throughout the life span of the host, and remain latent in reservoirs (Chesebro et al., 1979; Trono et al., 2010).

In conclusion, the FV mouse model represents a strong resource for the study of retroviral infection by providing an easily adaptable and versatile model in which all aspects of the immune response to retroviral infection can be investigated. Information gained from the study of the FV mouse model provides information that can further be applied to understanding other retroviral infections, such as HIV.

1.7 Lactate Dehydrogenase Elevating Virus

1.7.1 Lactate dehydrogenase elevating virus

Lactate dehydrogenase elevating virus (LDV) was first described in 1960, and was identified as a plasma enzyme-elevating virus (MAHY, 1964). LDV is a rapidly replicating RNA virus from the Nidovirales family, which cytopathically infects a subset of lactate dehydrogenase scavenging macrophages, causing an elevated level of lactate dehydrogenase in the host (Robertson et al., 2008). Infection with LDV occurs naturally in mice in the wild and although it does not have a harmful effect on the host, it has been shown to influence several aspects of the immune response, including reduction of antigen presentation by macrophages (Isakov et al., 1982), and activation of B cells leading to hypergammaglobulinaemia (Coutelier et al., 1990).

1.7.2 History

The possibility that previous studies and results regarding the immune response to FV may have been affected by contamination of the virus stock was largely ignored, despite a commentary on the subject several decades ago stating that LDV was a major contaminant in numerous transplantable murine tumours and suggesting that the absence of LDV should be confirmed for accuracy tumour studies (Riley, 1974). As well as transplantable tumour studies, LDV was also shown to increase the severity and duration of *Plasmodium yoelii* infection in a mouse model of malaria (Henderson et al., 1978).

1.7.3 Contamination of FV stocks

FV virus stock can be passaged *in vivo* or cloned *in vitro*, with *in vivo* passaging preferred due to its capacity to produce a virus complex containing more natural levels of SFFV due to positive selection of the virus (Robertson et al., 2008). However, *in vivo* passaging of FV resulted in contamination of virus stocks with LDV (Steeves et al., 1969). Despite this observation, researchers continued to use LDV-contaminated FV stock for several more decades. Coinfection of LDV and FV has been shown to have major effects on the immune response to FV, delaying recovery and increasing severity of FV disease. The effects of FV/LDV coinfection are discussed in detail below.

1.7.4 Effects of LDV coinfection on the immune response to FV

When FV alone infects immunocompetent and genetically resistant mice, the peak of infection occurs at day 7 and is subsequently resolved. However, in FV/LDV co-infection, infection does not peak by day 7 and continues to increase to a higher peak of infection at day 14, although again infection is resolved by day 28 (Marques et al., 2008).

As regards to effects on FV-specific immune responses, LDV has also been shown to have a suppressive effect on FV-specific $CD8^+$ T cells, resulting in a delayed $CD8^+$ T cell response in FV/LDV co-infected mice compared to FV alone, while no effect on the $CD4^+$ T cell response was observed. Further analysis of the mechanism of $CD8^+$ T cell-suppression showed that Treg cells induced by the virus are responsible for the delayed FV-specific $CD8^+$ T cell response in FV/LDV co-infection. The delay in peak

viraemia and recovery was attributed to the reduced $CD8^+$ T cell response during initial infection (Robertson et al., 2008).

In a separate study, B6 mice infected with FV alone did not develop splenomegaly and only marginal splenomegaly was seen after FV/LDV co-infection. In contrast, B6.A-*Fv2*^{s} mice, which have been shown to suffer only mild splenomegaly during FV infection alone, suffered from more severe splenomegaly during FV/LDV co-infection, with a delay in recovery similar to that described above. However, LDV coinfection did not alter the distribution of FV infection. Mice deficient in FV-specific B cells, have a more severe disease in both circumstances, and transfer of FV nAb was sufficient to reduce FV infection in both FV-infected mice and FV/LDV-co-infected mice. A delay in induction of FV nAb in FV/LDV co-infection was observed. This relied on the presence of a polyclonal B cell population since the effect was simulated by polyclonal activation of B cells (Marques et al., 2008). Thus, it can be concluded that polyclonal B cell activation by LDV results in delayed FV nAb response and that this, along with a delayed $CD8^+$ T cell response, contributes to increased severity of FV disease during FV/LDV coinfection.

1.7.5 The effect of FV/LDV co-infection on previous studies

The above described effects on LDV the pathogenesis of FV disease and the immune response to FV were largely overlooked in many previous studies and therefore must be taken into consideration when considering studies regarding the immune response to FV.

1.8 The Immune Response to FV

Ultimate control and clearance of FV infection depends on multiple facets of the adaptive immune response, and spontaneous relapse of disease can occur (Hasenkrug and Dittmer, 2000; Dittmer et al., 1999; Chesebro et al., 1974). Depletion, adoptive transfer and gene knock-out studies have attempted to reveal the individual roles and importance of different lymphocyte subsets. The FV-specific immune response has been studied using several methods beyond FV infection, including peptide immunisation, live attenuated virus vaccine, immunisation with the FBL-3 FV-induced leukaemia cell line, and retroviral vector vaccines. However, it must be considered that these observations may have been affected by LDV contamination of FV stocks.

1.8.1 The role of adaptive lymphocytes against FV infection

Two studies have highlighted the roles of $CD8^+$ and $CD4^+$ T cells at different stages of FV infection. A correlation between $CD8^+$ T cell cytotoxic activity and recovery of *H-2D*$^{b/b}$ mice from FV-induced splenomegaly has been observed. Furthermore, depletion of $CD8^+$ T cells by mAb during acute FV infection prevented recovery of splenomegaly. However, mice depleted of $CD4^+$ T cells during acute infection began to recover but disease was subsequently reactivated, with mice developing splenomegaly and erythroleukaemia. It was concluded from this study that $CD8^+$ T cells were required for recovery from initial disease while $CD4^+$ T cells were required to maintain control of chronic infection (Robertson et al., 1992).

In a complementary experiment, depletion of $CD4^+$ T cells by mAb during chronic FV infection in immunocompetent and otherwise resistant mice resulted in splenomegaly, erythroleukaemia and increased levels of viral replication. Conversely, depletion of $CD8^+$ T cells during chronic FV infection had no effect on viral load and did not lead to reactivation of disease. (Hasenkrug et al., 1998). Together these data show a requirement for $CD8^+$ T cells in controlling the initial FV infection and $CD4^+$ T cells for control of chronic persistent FV infection.

A constitutive $CD8^+$ T cell deficiency did not confer susceptibility to initial FV-induced disease in *Fv2*^{r} B6 mice, but did render mice susceptible to onset of late splenomegaly, although some of these animals recovered. Furthermore, $CD4^+$ T cell deficiency did not prevent control of acute infection by *Fv2*^{r} mice, but these mice could not maintain control and eventually succumbed to splenomegaly and erythroleukaemia with no recovery, further validating results from $CD4^+$ T cell depletion described above. This study suggests that both $CD4^+$ and $CD8^+$ T cells are required for prevention of late onset splenomegaly, but are not required to control the acute infection. Although B cell-deficient mice also showed increased susceptibility to late onset splenomegaly, this was mild compared to $CD4^+$ and $CD8^+$ T cell deficiencies (Hasenkrug, 1999). The findings of these studies are summarised in **Figure 1.4.**

Depletion of T cells in genetically resistant mice has been shown to result in susceptibility to FV disease and thus overcomes genetic resistance. Therefore, resistance to FV infection is mediated by both genetic and immunological factors.

However, processes that compensate for lymphocyte deficiencies exist, resulting in abnormal distribution of the remaining lymphocyte subsets, as evidenced by the increased percentage of $CD8^+$ T cells and a reduced percentage of B cells seen in $CD4^+$ T cell-deficient mice (Hasenkrug, 1999).

While the experiments described above have lead to the conclusion that $CD4^+$ T cells are required to control chronic infection while $CD8^+$ T cells are necessary for control of acute infection, others have suggested that $CD4^+$ and $CD8^+$ T cells are essential for preventing onset of splenomegaly during the chronic phase of infection, but play no role in reducing acute infection. These inconsistencies are likely to due to the presence of absence of LDV contamination and its effects on the FV-specific immune response, and further work to establish the precise roles of T lymphocytes during different stages of FV infection using a clean FV stock is therefore required.

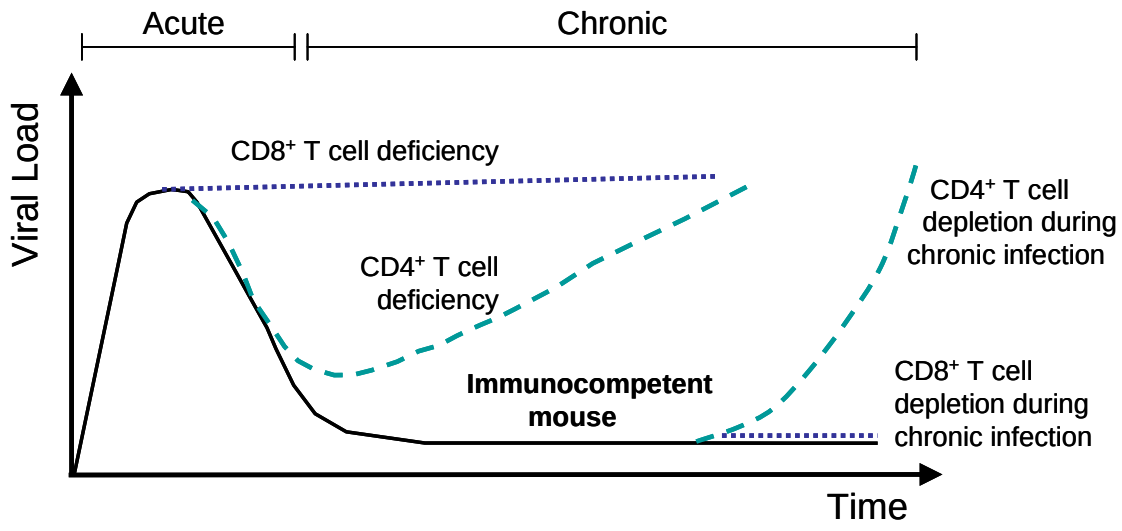

Figure 1.4 Pathogenesis of Friend virus infection and the effect of lymphocyte subset depletion

Pathogenesis of Friend virus infection in an immunocompetent mouse (uninterrupted line), and the effect of constitutive deficiencies or active depletion of $CD4^+$ T cells (dashed line) or $CD8^+$ T cells (dotted line) on viral load during the acute or chronic phase of the infection.

1.8.2 Cytotoxicity during FV infection

The ability of $CD8^+$ T cells to kill virally infected cells is well-described, and depletion of $CD8^+$ T cells has been shown to prevent recovery of FV-induced splenomegaly in mice (Robertson et al., 1992; Hasenkrug, 1999). With this in mind, functional activity of cytotoxic $CD8^+$ T cells in the FV model has also been investigated. Here, mice deficient in the Fas/FasL pathway were still able to control splenomegaly during acute FV infection but could not control persistent infection, although knock-out mice did not develop erythroleukaemia. Mice deficient in either perforin, granzyme A or granzyme B, or two of the three, were able to control FV infection in the presence of functional Fas/FasL, while deficiency of all three components rendered mice susceptible to FV-induced erythroleukaemia (Zelinskyy et al., 2004). Additionally, $CD8^+$ T cells *ex vivo* from infected mice did not show high cytotoxic activity, while previous studies have shown detectable cytotoxic activity of $CD4^+$ T cells, suggesting that they may be the major killers of infected cells during persistent FV infection (Iwashiro et al., 2001b).

1.8.3 Cytokine responses

As in all pathogen-specific immune responses, many cytokines work to control FV infection. Interferons are the major anti-viral cytokines, while other cytokines have important direct effects or play a role in modulating the activities of both innate and adaptive immune cells.

Type I interferons IFN-α and IFN-β were shown to inhibit replication of F-MuLV *in vitro*, with both cytokines induced soon after FV infection. In mice deficient in either the type I IFN receptor or IFN-β, viral load in the spleen was increased during both acute and chronic infection. IFN-α was shown to have a more profound effect, and administration of exogenous IFN-α before challenge was able to reduce FV infection in the acute phase by direct inhibition of viral replication and modulation of T cells (Gerlach et al., 2006). However, an author's correction was published stating that FV in fact does not induce type I interferons, and that the induction of IFN-α and IFN-β was caused by LDV contamination of FV stock . However, mRNA expression of IFN-α was detected in splenocytes of FV-infected animals, and the IFN-α subtype mediating this suppression was further investigated. Several subtypes of IFN-α were shown to be expressed in splenocytes, of which IFN-α1, α4 and α9 subtypes were able to reduce FV infection, with IFN-α4 being the most effective. While IFN-α4 and α9 mediated this effect via activation of NK cells, IFN-α1 also activated $CD8^+$ T cells (Gerlach et al., 2009).

IL-4, IL-12 and IFN-γ and their roles in the FV-specific immune response have also been investigated. While IFN-γ-deficient mice could not control acute FV infection,

IL-4- and IL-12-deficient mice did not have significantly higher viraemia than that seen in wild-type mice. Furthermore, some IFN-γ-deficient mice went on to develop splenomegaly during later disease, in contrast to IL-$4^{-/-}$, IL-$12^{-/-}$ or control mice. In addition, vaccination with the F-MuLV live attenuated virus vaccine was able to protect mice deficient in IL-4, IL-12 or IFN-γ. It was also shown that although FV nAb were induced in IFN-γ deficient mice, these mice had a compromised ability to class switch from IgM to IgG, and so only had IgM nAb. However, this did not affect live attenuated virus vaccine-mediated protection against FV (Dittmer et al., 2001).

Use of an env-specific $CD4^+$ T cell clone *in vitro* showed that IFN-γ had a direct inhibitory effect on virus production which required T cell activation, but not antigen presentation to $CD4^+$ T cells. This $CD4^+$ T cell clone was also shown to have cytotoxic activity which was dependent on antigen presentation, although it did not require MHC II expression by target cells, and was enhanced by IFN-γ. While IFN-γ-deficient B6 mice did not suffer from splenomegaly initially, they could not control persistent infection and succumbed to late onset splenomegaly (Iwashiro et al., 2001b). Further studies of the role of IFN-γ in FV infection found contradicting results. IFN-γ deficient mice had reduced levels of infection in the blood and bone marrow but not spleen at one week post infection. However, by 2 weeks post infection mice deficient in IFN-γ actually had a decreased level of infection in the spleen and bone marrow compared to wild-type mice, and were shown to have an increased level of nAb. However, later in FV infection IFN-γ-deficient mice had a lower FV nAb titre than wild-type mice, and could not prevent splenomegaly (Stromnes et al., 2002).

Alternatively, IFN-γ has been shown to inhibit formation of murine erythroid colonies and therefore IFN-γ could be also be having a direct effect in reducing FV infection, by controlling the proliferation of erythroid precursors induced by gp55 agonism of the EpoR during FV infection (Means, Jr. et al., 1994).

Other cytokines have also been studied in the context of FV infection, in particular IL-5 which can stimulate B cell growth, IL-6 which can stimulate B cell maturation, and the immunosuppressive cytokine IL-10. While IL-5-deficient mice did not have higher levels of infection, viral load was increased in the absence of IL-6 or IL-10 during the acute phase of infection, but control of persistent infection was not altered compared to wild-type mice. Additionally, vaccination with a live attenuated virus vaccine was still protective even in the absence of each cytokine individually (Strestik et al., 2001).

1.8.4 Regulation of the immune response to FV

During chronic FV infection regulatory $CD4^+$ T cells act to suppress adaptive immune responses, disabling clearance of latent infection but preventing immunopathology. Several studies have shown that this effect is mainly mediated by suppression of $CD8^+$ T cells, and these are described here.

Persistently FV infected mice were shown to have a marked expansion of $CD4^+$ T cells, while *in vitro*, $CD8^+$ T cell mediated cytotoxicity of murine leukaemia tumour cells was shown to be impaired by the presence of $CD4^+$ T cells from FV infected mice, but not from uninfected mice. This was further confirmed *in vivo*, and the number of $CD4^+$ T cells expressing regulatory T cell markers was shown to be increased in the expanded $CD4^+$ T cell population during chronic infection. The suppressive effect of these cells was shown to be mediated by the cytokine TGF-β and the cell surface molecule CTLA-4, but not the signature immunosuppressive cytokine IL-10 (Iwashiro et al., 2001a).

When TCR transgenic FV-specific $CD8^+$ T cells were transferred into persistently infected mice, the cells proliferated substantially and became activated compared to cells transferred into uninfected mice. Despite activation, these $CD8^+$ T cells did not produce IFN-γ and were not able to lower viral load. In contrast, TCR transgenic $CD8^+$ T cells transferred into acutely infected mice were able to lower viral load. Furthermore, specific inhibition of Treg-mediated immunosuppression *in vivo* restored IFN-γ-production by these $CD8^+$ T cells and reduced viral load, showing that

immunosuppression by Tregs is responsible for compromised $CD8^+$ T cell responses during chronic FV infection (Dittmer et al., 2004).

As shown above, Treg-mediated suppression reduces IFN-γ-production by $CD8^+$ T cells during chronic infection, abrogating this effector function. Further studies have examined cytotoxic activity of $CD8^+$ T cells during FV infection. *In vivo* cytotoxicity assays showed that cytotoxic $CD8^+$ T cells were active against tumour cells during acute infection, but not during chronic infection. It was further shown that activated FV-specific $CD8^+$ T cells express perforin, granzyme A and granzyme B during acute FV infection, but not during chronic infection, where there was a deficiency of these cytotoxic mediators. This was consistent with high expression of a degranulation marker on $CD8^+$ T cells isolated from acutely infected mice but a reduced expression on those isolated from chronically infected mice (Zelinskyy et al., 2005). Previous studies support the conclusion that Tregs are also mediating suppression of $CD8^+$ T cell cytotoxicity in FV infection, and several further reports have confirmed this (Myers et al., 2009; Zelinskyy et al., 2009a; Zelinskyy et al., 2009b)

1.8.5 Inducing a protective immune response against FV

Live attenuated virus vaccination of *Fv1*^{b} mice with N-tropic F-MuLV alone, which is non-pathogenic in mice of this genotype, was shown to be able to protect against challenge by B-tropic FV complex. The vaccine was also shown to induce virus-neutralising Ab, and whole immune spleen cells from these vaccinated mice conferred protection when transferred into naive mice, showing that protection occurs via an immunological mechanism and not through viral interference (Dittmer et al., 1998).

Increasing the number of whole immune spleen cells transferred has been shown to further increase the level of protection against FV infection, leading to an interest in the role of individual lymphocyte subsets. Transfer of immune B cells or $CD4^{+}$ T cells was not able to reduce viraemia, and all mice became splenomegalic upon infection with FV. Transfer of $CD8^{+}$ T cells reduced initial viraemia and aided recovery from splenomegaly but was not adequate to prevent initial FV infection or virus persistence. When a single lymphocyte subset was absent, mice were not protected from initial infection, but did recover from splenomegaly, while transfer of the three purified subsets remixed did confer protection. Additionally, transfer of nAb alone also reduced virus but did not clear it, and this effect was dependent on the presence of non-immune T cells. Thus, all three adaptive lymphocyte subsets appear to be necessary for the protection induced by a live attenuated F-MuLV vaccine (Dittmer et al., 1999).

Transfer of immune $CD8^{+}$ T cells and passive immunisation with FV neutralising mAb permitted $CD4^{+}$ T cell-dependent recovery from splenomegaly in mice.

However, transferring increased numbers of each immune lymphocyte subset showed that even higher numbers of immune $CD4^+$ T cells or immune B cells were not sufficient to protect mice against splenomegaly, while adoptive transfer of higher numbers of immune $CD8^+$ T cells further reduced the incidence of splenomegaly in mice compared to that seen after transfer of lower numbers. Increasing virus-neutralising mAb dose was also able to further reduce splenomegaly. The amount of free virus was also reduced by additional immune $CD8^+$ T cells or nAb. It was further confirmed that all three adaptive lymphocyte subsets were required for protection against FV persistence: $CD8^+$ T cells for infected cell killing, B cells for production of virus-nAb, and $CD4^+$ T cells for immunological help to $CD8^+$ T cells and B cells (Dittmer and Hasenkrug, 2000).

Transfer of FBL-3 cells from the FBL-3 murine leukaemia-derived cell-line, was not able to induce virus-specific nAb, but does induce a strong $CD8^+$ T cell response which is still protective in the absence of B cells, suggesting that B cell priming of $CD8^+$ T cells is not required for their function. While live attenuated virus vaccine can protect in the presence, but not absence of B cells, administration of nAb restores this protection and nAb alone is sufficient to reduce viraemia. The protection seen by a live attenuated virus vaccine was reproduced by administration of a $CD8^+$ T cell-inducing FBL-3 vaccination and FV nAb with the protective effect evident in the first few days after infection. Non-neutralising Ab had no effect alone but was able to increase the effect of virus-nAb in the absence of a $CD8^+$ T cell response (Messer et al., 2004). This essential role for nAb is also demonstrated during FV/LDV co-infection as described above, where the delayed induction of nAb production contributes towards prevention of recovery from FV disease. (Marques et al., 2008).

1.8.6 Inducing FV-specific $CD4^+$ T cells

Several efforts to assess the effect of increasing FV-specific $CD4^+$ T cells on FV infection have been inconclusive. Immunisation with an F-MuLV env peptide recognised by $CD4^+$ T cells, and complete Freund's adjuvant (CFA) showed varying results depending on the epitope used and the *H-2* genotype-determined susceptibility of the mice. Immunisation with the N-terminal epitope, which is presented in complex with the A^b class II molecule in $H\text{-}2^{a/b}$ mice, was able to protect from late onset splenomegaly, while immunisation with the C-terminal epitope, which is presented in complex with the $E^{b/d(k)}$ class II molecule, cleared virus and prevented onset of early splenomegaly in $H\text{-}2^{a/b}$ mice. In contrast, $H\text{-}2^{a/a}$ mice were not protected by either peptide. Purified F-MuLV virus particles, however, were able to protect against FV infection in both $H\text{-}2^{a/b}$ and $H\text{-}2^{a/a}$ mice. Furthermore, peptide-immunised $H\text{-}2^{a/b}$ mice showed accelerated development and class-switching from IgM to IgG FV nAb after FV-infection. However, reduction of infection was seen in the spleen before induction of FV nAb, suggesting that the protective effect demonstrated after peptide induction of $CD4^+$ T cells occurred via an alternative mechanism, as well as helping production of nAb (Miyazawa et al., 1995).

The mechanisms by which this peptide immunisation conferred protection have been subsequently analysed. Cytotoxicity assays showed that cells in the spleen exerted cytotoxic activity against FV-induced leukaemia cells, and separation of $CD4^+$ cells, $CD8^+$ cells or $CD4^-CD8^-$ cells revealed that all three populations had cytotoxic activity after peptide immunisation. Within the $CD4^-CD8^-$ effector population, cytotoxic activity was higher than that seen in the $CD4^+$ or $CD8^+$ populations. Furthermore, NK cells were shown to encompass 10-30% of these $CD4^-CD8^-$ cells

and depletion of NK cells both reduced the cytotoxic activity of the $CD4^-CD8^-$ population in an FV-specific manner, and abrogated the protection against erythroleukaemia provided by peptide immunisation (Iwanami et al., 2001).

1.8.7 FV-mediated immunosuppression

FV has been shown to cause immunosuppression in susceptible mice, suppressing both cellular and humoral responses (Hasenkrug and Chesebro, 1997). FV infection of mice has been reported to suppress antibody development in response to immunisation with sheep erythrocytes as well as reducing the number of antibody precursors (Ceglowski and Friedman, 1968; Ceglowski and Friedman, 1970). Migration of immune cells was also shown to be inhibited *in vitro* (Friedman and Ceglowski, 1971). Other murine leukaemia viruses have also been shown to alter DC function, altering antigen presentation during retroviral infection (Gabrilovich et al., 1994).

However, much of this work was done with FV stocks contaminating with LDV, resulting in FV/LDV coinfection. As discussed above, LDV has profound effects on the immune response, and potentially the observed immunosuppression during FV infection is not mediated by FV, but by LDV. A more recent study has shown that direct infection of myeloid DCs with FV inhibits their development and function, and results in expansion of Treg cells (Balkow et al., 2007). Although LDV was not discussed in the context of this study, the date of publication suggests that this paper was published when LDV contamination of FV stocks had been acknowledged by the

authors. The potential immunosuppressive activity of FV should therefore be reassessed using an uncontaminated FV stock.

1.8.8 Validity of previous studies

It is clear that the effects of potential LDV contamination of FV stocks were overlooked in the studies described above. This may explain why results have been varied and ultimately inconclusive. Hence it is likely that conclusions from the above studies do not provide an accurate depiction of the immune response to FV. While these studies may provide a basis for the study of the immune response to FV, it is necessary to re-establish the roles of different lymphocyte subsets and cytokines in the immune response against the virus.

1.9 Re-assessing the Role of $CD4^+$ T Cells against FV

While many previous studies have concluded that the role for $CD4^+$ T cells is predominantly during the chronic phase of FV infection, several others have described a role for $CD4^+$ T cells in the immune response to acute FV infection. LDV contamination of FV stocks and the subsequent effects of LDV co-infection on the FV-specific immune response lead us to re-examine the role of $CD4^+$ T cells against FV infection.

1.9.1 The role of $CD4^+$ T cells in primary FV infection

In order to ascertain whether $CD4^+$ T cells play a role in the control of acute FV infection, B6-*MHC II*$^{-/-}$ mice, which have no endogenous $CD4^+$ T cells, were infected with FV and the percent of infected erythroid precursor cells in the spleen was detected by flow cytometry at the peak of infection. Mice deficient in $CD4^+$ T cells were shown to have significantly more infected cells in the spleen than WT mice, showing that $CD4^+$ T cells do indeed contribute towards protection in the primary immune response against FV infection (**Figure 1.5**) (work carried out by Andrew Filby). With this in mind, a transgenic mouse was developed in which to further examine the role of $CD4^+$ T cells against FV infection.

It was shown that the level of infected cells in the spleen at the peak of infection was significantly increased in B6-*MHC II*$^{-/-}$ mice, which are deficient in $CD4^+$ T cells, compared to immunocompetent mice after infection with a clean LDV-free FV stock. This provided evidence that $CD4^+$ T cells have a role against FV infection during the acute phase, and that infection is augmented in their absence.

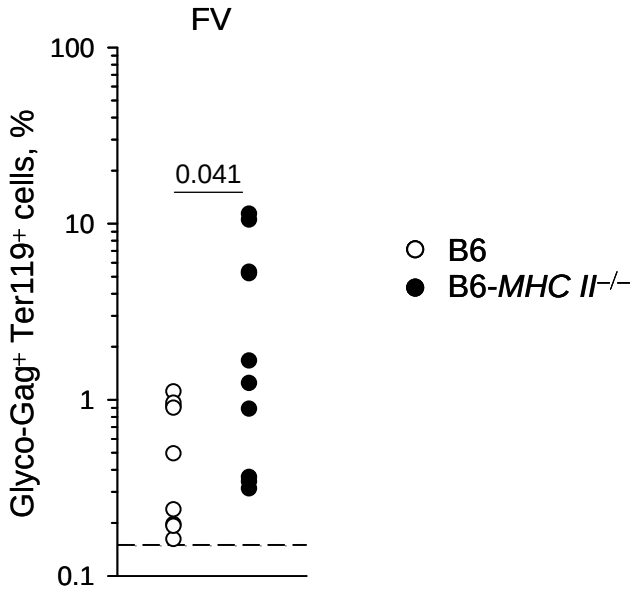

Figure 1.5 The contribution of endogenous CD4$^+$ T cells to protection against acute FV infection (work carried out by Andrew Filby)

MHC class II-deficient (B6-*MHC II*$^{-/-}$) and control B6 mice were infected with FV, and the percentage of FV-infected (glyco-Gag$^+$) Ter119$^+$ cells in the spleen 7 days post infection is shown. Each symbol represents an individual mouse. The dashed line indicates the limit of flow cytometric detection. The number denotes the *p* value as compared by the two-tailed Wilcoxon-Mann-Whitney test.

1.9.2 The EF4.1 TCRβ-transgenic mouse

In addition to LDV contamination of FV stocks, another major caveat associated with previous studies was the lack of method by which to quantify the FV-specific $CD4^+$ T cell response, analyse the function or quality of FV-specific $CD4^+$ T cells, or even to detect whether FV-specific $CD4^+$ T cells had been induced at all.

In order to quantify and quantitate the FV-specific $CD4^+$ T cell response, and thus overcome the above described caveat, a mouse strain expressing a transgenic $CD4^+$ T cell TCRβ chain was generated in our laboratory. This TCRβ chain was derived from a clone (clone SB14-31) that was isolated from an FBL-3-challenged mouse and was shown to be reactive to the N-terminal region of the gp70 env protein of FV encompassing the amino-acid residues 122-141 ($env_{122\text{-}141}$): DEPLTSLTPRCNTAWNRLKL (Iwashiro et al., 1993). These mice are referred to as EF4.1 TCRβ-transgenic mice and were created as previously described (Antunes et al., 2008).

During $CD4^+$ T cell development in EF4.1 mice, the transgenic TCRβ chain pairs with endogenous TCRα chains, resulting in a polyclonal $CD4^+$ T cell population. Flow cytometric staining of CD69 and CD40L as markers of $CD4^+$ T cell activation (**Figure 1.6A**) after *in vitro* stimulation of EF4.1 TCRβ-transgenic $CD4^+$ T cells with F-MuLV $env_{122\text{-}141}$ peptide revealed that 4% of this polyclonal population upregulated these markers and hence were specific for F-MuLV $env_{122\text{-}141}$ (**Figure 1.6B**).

A panel of antibodies was tested to identify preferential use of particular Vα TCR chains by the FV-specific $CD4^+$ T cell population. Vα2 was identified as the TCR chain that conferred specificity to FV, with 15% of the $Vα2^+$ population responding to $env_{122-141}$, while only 3% of the $Vα2^-$ population responded to *in vitro* stimulation by the peptide. When the response of $Vα2^+$ env-specific $CD4^+$ T cells was compared to $Vα2^-$ env-specific $CD4^+$ T cells, the $Vα2^+$ population was shown to be more than 30 times more sensitive to $env_{122-141}$ (**Figure 1.6C**) (Antunes et al., 2008). (All work in section 1.9.2 was carried out by Inês Antunes.)

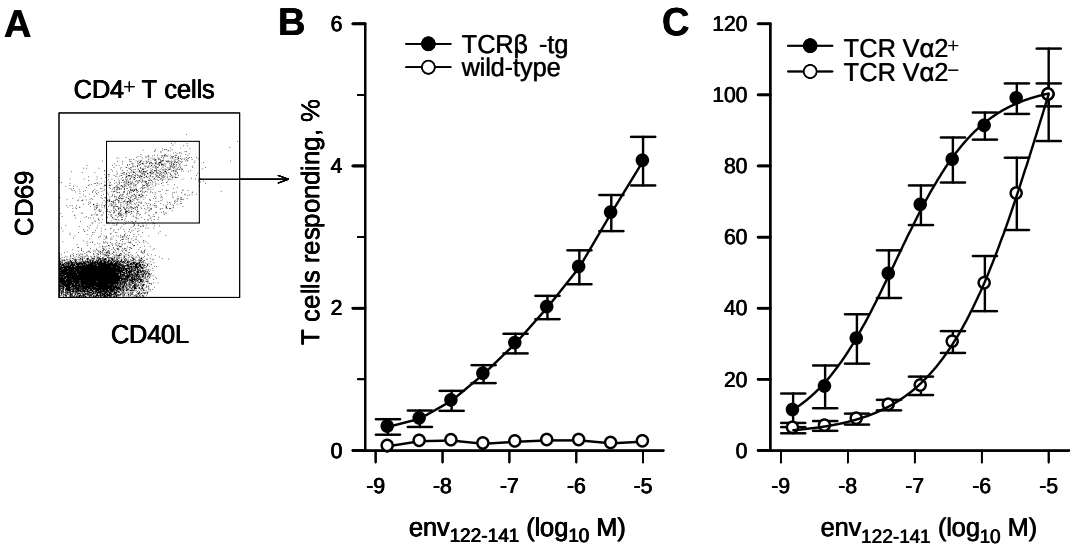

Figure 1.6 T cell reactivity to $env_{122-141}$ and TCR Vα2 usage in EF4.1 TCRβ-transgenic mice (Antunes et al., 2008) (Work carried out by Inês Antunes)

(A) Flow cytometric example of CD69 and CD40L induction in $CD4^+$ EF4.1 TCRβ-transgenic T cells 18 hr after stimulation with the $env_{122-141}$ peptide.

(B) Mean (± the SEM) percentage of responding cells in total $CD4^+$ T cells from EF4.1 TCRβ-transgenic or wild-type control mice 18 hr after stimulation.

(C) Mean (± the SEM) percentage of responding cells, plotted as a fraction of the maximal response, in gated TCR $Vα2^+$ or $Vα2^-$ $CD4^+$ T cells from EF4.1 TCRβ-transgenic mice 18 hr after stimulation.

1.9.3 Clonal composition of EF4.1 TCRβ-transgenic CD4$^+$ T cells in response to FV infection

High avidity CD8$^+$ T cells have been shown to be more protective than those of low avidity in several studies (Alexander-Miller et al., 1996; Sedlik et al., 2000). This suggests that an FV-specific CD4$^+$ T cell response dominated by high avidity cells would in theory be more protective than low avidity. The clonal composition of responding FV-specific cells was therefore examined.

The response of TCRβ-transgenic CD4$^+$ T cells to FV infection can be followed, and the clonal composition of the FV-specific CD4$^+$ T cell population studied using flow cytometry by transferring CD45.1 EF4.1 TCRβ-transgenic CD4$^+$ T cells into B6 (CD45.2) mice. By staining for surface markers including CD4 to identify the CD4$^+$ T cell population, CD45.1 to identify the TCRβ-transgenic donor cell population, CD44 to identify the antigen-experienced cells, and the Vα2 TCR chain, it was possible to study the clonal composition of the responding FV-specific CD4$^+$ T cell population. FV-specific cells are gated as CD4$^+$CD45.1$^+$CD44^{hi}.

At the peak of FV infection (day 7) approximately 10% of cells in the endogenous CD4$^+$ T cell population used the Vα2 TCR chain. In the antigen-naïve donor CD4$^+$ T cell population, approximately 12% of cells used the Vα2 TCR chain, while in the responding donor CD4$^+$ T cell population there was an enrichment of high avidity Vα2$^+$ CD4$^+$ T cells, expanding to encompass over 60% of all F-MuLV env-specific CD4$^+$ T cells (Ploquin et al., manuscript submitted) (**Figure 1.7**).

This massive expansion of high avidity Vα2$^+$ CD4$^+$ T cells was followed by their dramatic loss, and by day 35 after infection the ratio of Vα2$^+$ cells to Vα2$^-$ cells was the same as that seen in the antigen naive population (**Figure 1.8A**). This preferential expansion during the peak of infection and increased rate of loss of high avidity Vα2$^+$ CD4$^+$ T cells was also evident when the number of Vα2$^+$ or Vα2$^-$ FV-specific CD4$^+$ T cells during the course of infection was plotted, showing that Vα2+ cells are selectively lost and that this observation was not merely due to overall loss of overall FV-specific CD4$^+$ T cells (Ploquin et al., manuscript submitted) (**Figure 1.8B**).

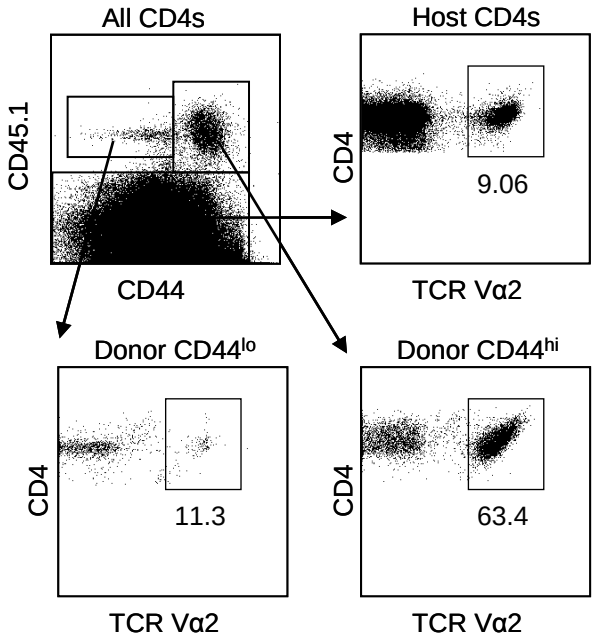

Figure 1.7 Flow cytometric example of expression of Vα2 on CD4$^+$ T cells (Ploquin et al., manuscript submitted) (Work carried out by Andrew Filby)

Flow cytometric example of expression of Vα2 on CD44^{hi} donor TCRβ-transgenic CD4$^+$ T cells, CD44^{lo} donor EF4.1 CD4$^+$ T cells and the endogenous CD4$^+$ T cell population in mice which received TCRβ-transgenic CD4$^+$ T cells and were FV infected 1 day later.

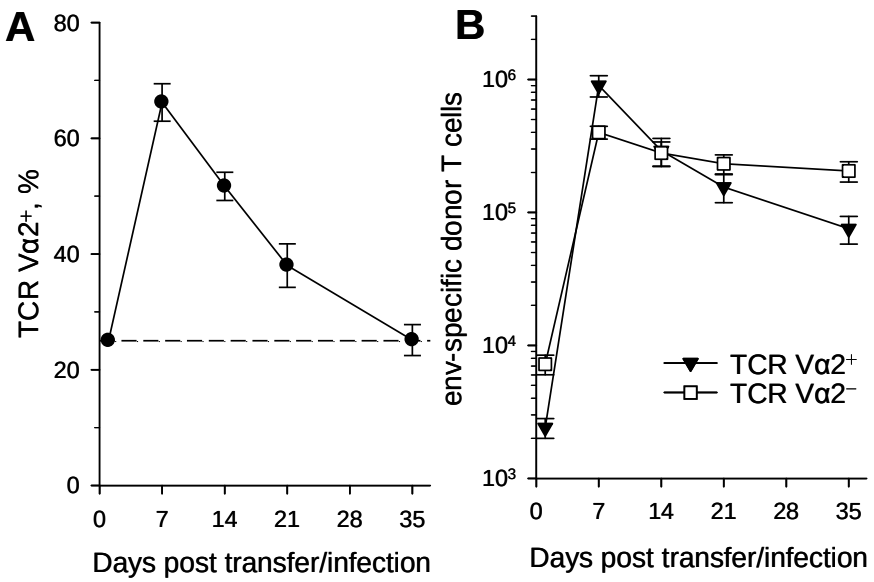

Figure 1.8 Clonal composition of FV-specific TCRβ-transgenic CD4$^+$ T cells *in vivo* during FV infection (Ploquin et al., manuscript submitted) (Work carried out by Andrew Filby)

(A) Frequency of env-specific CD4$^+$ T cells expressing TCR Vα2 in mice which received 1 × 10^6 EF4.1 TCRβ-transgenic CD4$^+$ T cells and were FV infected. Values are the mean (± the SEM). The dashed line indicates the percent of Vα2$^+$ cells seen in the naïve EF4.1 TCRβ-transgenic CD4$^+$ T cell population.

(B) Numbers of Vα2$^+$ and Vα2$^-$ FV-specific CD4$^+$ T cells recovered from mice after FV infection. Values are the mean (± the SEM).

1.10 Aims and Objectives

The aim of the work described in this thesis is to re-evaluate and further establish the role of $CD4^+$ T cells against retroviral infection, using the FV complex as a mouse model of retroviral infection and the established $CD4^+$ T cell TCRβ-transgenic B6.EF4.1 mouse (Antunes et al., 2008). In contrast to previous studies investigating the $CD4^+$ T cell response to FV which may have been marred by the presence of FV/LDV coinfection, experiments in this thesis used an uncontaminated FV stock.

Additionally, and again in contrast to previous studies, it will be possible to identify, quantify and monitor the FV-specific $CD4^+$ T cell response due to the availability of the EF4.1 TCRβ-transgenic mouse. This will also facilitate examination of the functional quality and clonal composition of FV-specific $CD4^+$ T cells in the response to FV infection and vaccination techniques. Furthermore, the mechanisms underlying the loss of high avidity $V\alpha2^+$ FV-specific $CD4^+$ T cells are examined and ways in which to induce and preserve an FV-specific memory $CD4^+$ T cell pool dominated by high avidity $V\alpha2^+$ $CD4^+$ T cells are investigated.

Chapter Two

2 Materials and Methods

2.1 Mice

Mouse strains used in the experiments are listed in **Table 2.1** and were maintained at the National Institute for Medical Research (NIMR) animal facilities. All animal experiments were conducted according to the Home Office regulations and local guidelines.

Common Name	Strain Name	Targeted Gene	Source/Reference
WT mice			
B6	C57BL/6		Jackson Laboratory (Bar Harbor, Maine, USA)
Congenic			
B6.CD45.1	B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ		Jackson Laboratory (Bar Harbor, Maine, USA)
B6.*Fv1*^{n}	B6.C3H-*Fv1*^{n}	*Fv1*	(Pike et al., 2009)
B6.*Fv2*^{s}	B6.A-*Fv2*^{s}	*Fv2*	(Marques et al., 2008)
Targeted Mutants			
Rag1$^{-/-}$	B6.129S7-*Rag1*^{tm1Mom}/J	*Rag 1*	(Mombaerts et al., 1992)
IFNγR1$^{-/-}$	B6.129S7-*Ifngr1*^{tm1Agt}/J	*Ifngr1*	(Huang et al., 1993)
µMT or *Igh6*$^{-/-}$	B6.129S7-*Igh-6*^{tm1Cgn}/J	*Igh6*	(Kitamura et al., 1991)
B6.*Fv2*^{s} *Rag1*$^{-/-}$	B6.A-*Fv2*^{s} *Rag1*$^{-/-}$	*Rag 1*, *Fv2*	(Marques et al., 2008)
Transgenic			
EF4.1 TCRβ-transgenic	B6 EF4.1 TCRβ	*TCRβ*	(Antunes et al., 2008)
EF4.1 CD45.1 TCRβ-transgenic	B6 EF4.1 CD45.1 TCRβ	*TCRβ*	(Antunes et al., 2008)

Table 2.1 Name, targeted gene and source (or reference) of mouse strains used.

2.2 Flow Cytometry Analysis

2.2.1 Cell and tissue preparation

Animals were killed by an approved schedule I method. Spleen and lymph nodes (inguinal, axillary, brachial, mesenteric and superficial cervical) were isolated and single cell suspensions were prepared by mechanical disruption of organs through a 70 µm cell strainer (Falcon, Becton Dickinson Labware).

All cell suspensions were prepared and stored in Air-Buffered (AB) Iscove's Modified Dulbecco's Medium (IMDM) containing 25 mM HEPES buffer and L-glutamine and supplemented with

- 0.21% NaCl
- 60µg/ml penicillin
- 100µg/ml streptomycin

(all Invitrogen Life Technologies)

Spleen cells were subjected to erythrocyte lysis by treatment with ammonium–chloride-potassium (ACK) lysis buffer (pH 7.2-7.4) containing

- 0.15 M NH_4Cl
- 1mM $KHCO_3$
- 0.1mM EDTA

After erythrocyte lysis, cells were washed and resuspended in AB IMDM.

Cell numbers were determined using an automated cell counter (Casy1, Schaerfe Systems, Reutlinger, Germany) or a Neubauer haemocytometer (Marienfeld). Lymph node cellularity was calculated as the sum of the cellular contents of inguinal, axillary, brachial, superficial cervical and mesenteric lymph nodes. Total numbers of each cell type were calculated from the frequency determined by flow cytometry and the total number of cells recovered from each organ.

Blood was taken from mice by making a small incision of the tail vein and approximately 50 µl of blood was collected in tubes containing heparin or with heparinised capillary tubes.

2.2.2 Cell surface marker staining

Cell expression of surface antigens was measured by monoclonal antibody (mAb) staining of freshly isolated cells, followed by Fluorescence Activated Cell Sorter (FACS) analysis. Cell concentrations were adjusted to 1×10^8 cells/ml for analysis. Cells were incubated with anti-FcR mAb (2.4G2) to block non-specific binding through Fc receptors, and stained with directly-conjugated antibodies to surface markers for 30 minutes. All stainings were performed in the dark and cells were washed and stained in FACS buffer containing

- PBS
- 2% FCS
- 0.1% azide

Antibodies used were obtained from eBiosciences, CALTAG/Invitrogen, BD Biosciences or prepared at NIMR and are summarized in **Table 2.2**.

Specificity	**Clone name**	**Company**
Fcγ III/II R	2.4G2	NIMR
CD4 (L3T4)	RM4-5	eBioscience / Caltag
CD25 (IL-2 receptor α chain)	PC61.5	eBioscience
CD43 (Ly-48; leukosialin)	1B11	BD Biosciences
CD44 (Pgp-1; H-CAM; Ly-24)	IM7	eBioscience
CD45.1 (Ly-5.1)	A20	eBioscience
CD45.2 (Ly-5.2)	104	eBioscience
CD122	5H4	eBioscience
CD127 (IL-7receptor α chain)	A7R34	eBioscience
Glyco-Gag	34	NIMR
Ter119/Erythroid cells (Ly-76)	TER-119	eBioscience
TCRVα2	B20.1	eBioscience
TCRVα2	B20.6	BD Biosciences
2nd layers		
mIgG2b-FITC	R12-3	BD Biosciences

Table 2.2 Specificity, clone name and source of anti-mouse antibodies used for extracellular staining. Alternative names are shown in parentheses.

Up to 4-colour samples were acquired on an analytical flow cytometer (FACSCalibur, BD Biosciences) and analyzed with FlowJo v8.7 software (Tree Star Inc). Up to 8-colour cytometry was performed on a CyAn flow cytometer (Dako, Fort Collins, CO) and analyzed with Summit v4.3 analysis software (Dako).

Figure 2.1 shows the gating strategy employed throughout to detect FV-specific donor $CD4^+$ T cells after adoptive transfer.

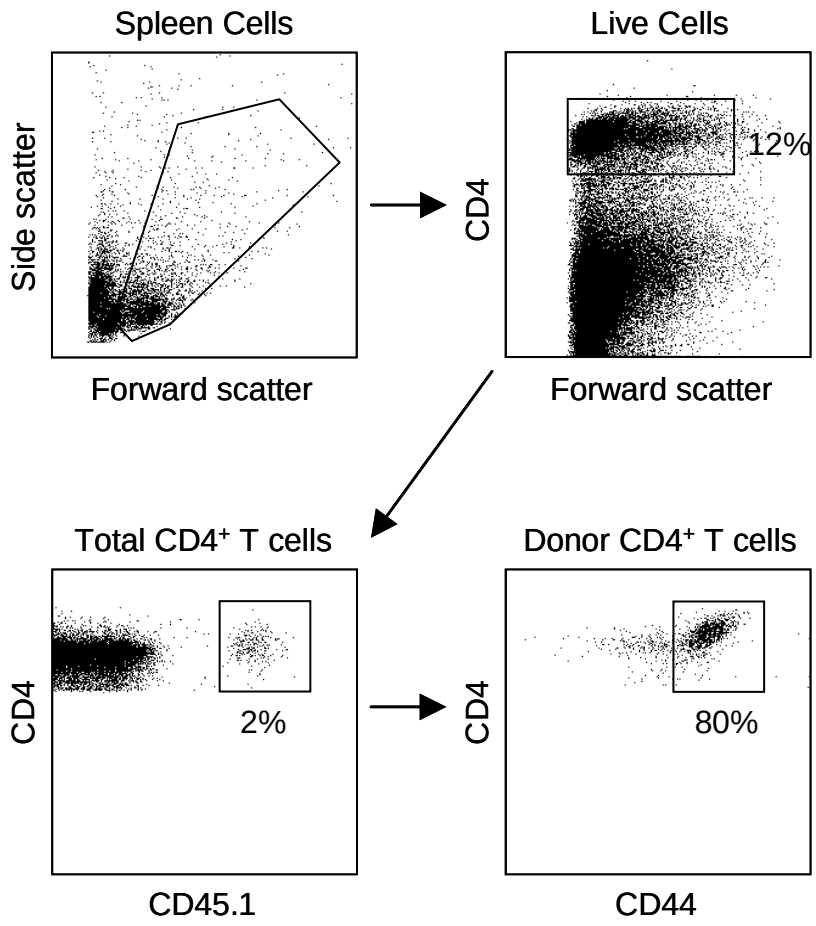

Figure 2.1 Gating strategy for identification of FV FV-specific donor CD4$^+$ T cells after adoptive transfer

Flow cytometric example of the gating applied to define the FV-specific donor (CD45.1$^+$) CD4$^+$ T cell population after adoptive transfer into B6 (CD45.2$^+$) recipients one day before FV infection. Cells were stained for surface antibodies and live cells were gated on size and granularity. CD4$^+$ T cells within the live cell population were gated and the percentage of cells collected is shown. Donor cells (CD45.1$^+$) are gated and donor cells within the total CD4$^+$ T cell population are shown. Responding (CD44^{hi}) donor CD4$^+$ T cells in FV-infected mice are gated, and activated, FV-specific donor CD4$^+$ T cells (CD44^{hi} CD45.1$^+$) are shown.

2.2.3 Intracellular cytokine staining

For intracellular cytokine staining, cytokine production was induced by stimulating cells with phorbol 12,13-dibutyrate (PdBu) and ionomycin (both at 0.5 µg/ml) together with surface staining antibodies in tissue culture conditions for 1 hour. After this period, an inhibitor of intracellular transport, monensin (at 1µg/ml), was added to block secretion of cytokines and cells were incubated for an additional 3 hours in tissue culture conditions. Cells were then washed and resuspended in fixation buffer (eBioscience) for 20 minutes at room temperature (RT) and then washed and incubated in permeabilisation buffer (eBioscience) with antibodies for cytokine staining for one hour at RT. Antibodies used for intracellular cytokine staining are summarised in **Table 2.3**.

Cytokines	Clone Name	Company
IL-2-PE	JES6-5H4	eBioscience
IL-2-PBlue	JES6-5H4	Insight Biotech
IL-17-FITC	eBioTC11-18H10.1	eBioscience
IFN-γ-PE	XMG1.2	Insight Biotech
IFN-γ-FITC	XMG1.2	eBioscience

Table 2.3 Specificity, clone, name and source of anti-mouse antibodies used for intracellular cytokine staining.

2.2.4 CFSE labelling

For carboxyfluorescein succinimidyl ester (CFSE) labelling, cells were washed in Dulbecco's Phosphate-Buffered Saline solution (D-PBS) (GIBCO) and resuspended in PBS with CFSE (Molecular Probes, Invitrogen). For labelling more than 1×10^6 cells CFSE was used at 2.5 µM, while for lower cell numbers the concentration was 0.6 µM. Cells were incubated for 10 minutes in tissue culture conditions and then washed in culture medium. Dividing cells were identified by CFSE dilution on FACS analysis.

2.3 Cell Purification and Sorting

2.3.1 Purification of $CD4^+$ cells

Target cells were enriched in lymph node and spleen suspensions using immunomagnetic positive selection (EasySep beads, StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. For $CD4^+$ T cell enrichment, single cell suspensions were diluted to a concentration of 1×10^8 cells/ml. Positive selection was then performed by staining the cells with CD4 PE-conjugated Ab for 30 minutes at 4^oC. Cells were washed, PE selection cocktail (25 µl/ml) was added and cells were incubated at RT for 15 minutes. Magnetic beads (25 µl/ml) were then added and cells were incubated at RT for a further 10 minutes. The tube containing the cells was placed on a magnet (EasySep Magnet, StemCell Technologies) for 5 minutes. The magnetic field allowed for retention of labelled cells, while the non-labelled cells remained in the supernatant. The supernatant was poured off and the tube was removed from the magnet. The walls of the tube, containing the selected cells, were washed with AB IMDM. After two additional selection steps, positively-selected cells were collected.

2.3.2 Cell sorting

For cell sorting, enriched cell suspensions were stained with antibodies for surface markers and then further purified by MoFlo cell sorters (Dako) by the NIMR Cell Sorting facility. Typical cell purity following cell sorting was higher than 98%. For example, for sorting of $V\alpha2^+$ and $V\alpha2^-$ cells for adoptive transfer, $CD4^+$ T cells were purified as described above and stained for CD45RB and TCR Vα2. $CD4^+/CD45RB^{hi}/V\alpha2^-$ or $CD4^+/CD45RB^{hi}/V\alpha2^+$ cells were sorted.

2.4 Adoptive Transfer, Infection and Immunisation

2.4.1 Adoptive transfer of T cells

$CD4^+$ T cells were isolated from spleen and lymph nodes of B6 EF4.1 TCRβ-transgenic or B6 EF4.1 CD45.1 TCRβ-transgenic donor mice with immunomagnetic positive selection (EasySep beads, Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions and as described above. In some experiments, cells were further isolated using cell sorting. Purified cells were injected in recipient mice via the tail vein in 0.1 ml of AB IMDM.

2.4.2 Friend virus infection

The Friend Virus (FV) used in these studies was a retroviral complex of a replication-competent B-tropic helper murine leukaemia virus (F-MuLV) and a replication-defective polycythemia-inducing spleen focus-forming virus (SFFV). The FV stock (kindly provided by Dr. Kim Hasenkrug, Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT, USA) was free of lactate dehydrogenase elevating virus (LDV) and was obtained as previously described (Robertson et al., 2008). FV was propagated *in vivo* and prepared as 10% w/v homogenate from the spleen of 12-day-infected BALB/c mice. Mice received an inoculum of ~1000 spleen focus-forming units (SFFU) of FV, injected via the tail vein in 0.1 ml of PBS.

2.4.3 Peptide immunisation

For peptide immunisation, $env_{124-138}$ (PLTSLTPRCNTAWNR) was injected with MPL adjuvant using the Sigma Adjuvant System (Sigma-Aldrich, Inc., Saint Louis, MO, USA). The Sigma adjuvant system is a stable oil-in-water emulsion. Each vial contains which contains 0.5 mg Monophosphoryl Lipid A (isolated from *Salmonella minnesota*) and 0.5 mg synthetic trehalose dicorynomycolate (an analogue of trehalose dimycolate from the cord factor of the tubercle bacillus) in 44 µl of squalene oil, 0.2% TWEEN 80 and water.

15 µM/mouse of peptide in solution was prepared according to the manufacturer's instructions. Briefly, Sigma Adjuvant System vial contents were warmed to 37^{o}C, 1 ml PBS was added and the vial was vortexed to produce an emulsion. Peptide diluted in PBS was added to the Sigma adjuvant solution in a 1:1 ratio and this was further vortexed to ensure even distribution of peptide within the emulsion. Peptide in adjuvant emulsion was kept at 37^{o}C and vortexed immediately before injection, and was injected intra-peritoneally (i.p.) in 0.15 ml.

2.5 Serum Preparation

Serum was prepared by leaving non-heparinised blood to clot at RT for 2 hours or at 4^{o}C overnight. The clot was then detached from the side of the eppendorf and the samples were centrifuged at 3,000 rpm for 5 minutes. Clear sera were transferred to new tubes and centrifuged at 12,000 rpm for 5 minutes. Clear sera were then transferred to new tubes, heat inactivated at 56^{o}C for 10 minutes and then stored at -20^{o}C.

2.6 FV Neutralizing Antibody Titre Assay

FV-neutralising antibodies in the sera of infected mice were measured using a modification of a previously described viral titre assay (Marques et al., 2008). *Mus. dunni* cells (Lander and Chattopadhyay, 1984) were transduced with the XG7 replication-defective retroviral vector, expressing GFP from a human cytomegalovirus (hCMV) promoter and a neomycin-resistance gene under the control of the LTR (Bock et al., 2000). (*Mus dunni* and *Mus dunni*-XG7 cells were both kindly donated by Dr Jonathan Stoye, Division of Virology, NIMR.) *M. dunni*-XG7 cells were then infected with F-MuLV-B and supernatant, which contained the pseudotyped XG7 vector, was harvested. Serial dilutions of sera from infected mice were mixed with ~1,500 infectious units (iu)/ml pseudotyped XG7 vector and allowed to incubate for 30 minutes at 37^{o}C in IMDM culture medium containing 5% FCS. Mixtures were then added to untransduced *M. dunni* cells and incubated for 3 days. The percentage of GFP^{+} *M. dunni* cells at the end of the incubation period was assessed by flow cytometry and the dilution of serum which resulted in 75% neutralisation (i.e. 75% reduction in the percentage of GFP^{+} *M. dunni* cells) was taken as the neutralising titre (Bock et al., 2000).

Culture medium which was used for the maintenance of cell lines and *in vitro* assays was IMDM (Sigma) supplemented with

- 5% heat inactivated FCS (BioSera)
- 2 mM L-glutamine (Sigma)
- 100 U/ml penicillin (Sigma)
- 100 µg/ml streptomycin (Sigma)

- 10^{-5} m mercaptoethanol (Sigma)

Tissue culture conditions were

- 95% humidity
- 5% CO_2
- 37^{o}C

2.7 Nucleic Acid Extraction

2.7.1 DNA extraction

Spleen cells were incubated for 3 hours with 0.5 ml lysis buffer consisting of

- 100 mM Tris-HCl (pH 8.5)
- 5 mM EDTA
- 200 mM NaCl
- 0.2% SDS
- 200 µg/ml of proteinase K (Roche Diagnostics GmBH)

Samples were vortexed and centrifuged at 12,000g for 10 mins and supernatant was transferred to a new tube. An equal amount of isopropanol was added and samples were mixed before spinning for 5 mins at 12,000g and washing with 70% ethanol. Pelleted DNA was air-dried and resuspended in clean water before being stored at -20^{o}C.

2.7.2 RNA extraction

RNA was extracted from cells by adding trizol (0.2 ml per 1 ml samples) followed by incubation at room temperature for 5 minutes. Samples were mixed and centrifuged at 12,000g for 15 minutes at 4^{o}C. The aqueous phase was transferred to a fresh tube and RNA was precipitated with isopropanol. Samples were centrifuged at 12,000g for 8 mins at 4^{o}C, pellets washed in 75% ethanol and centrifuged for a further 5 min at 12,000g and 4^{o}C. RNA pellets were resuspended in water and stored at -20^{o}C.

2.7.3 Synthesis of cDNA

30 µl of RNA resuspended in water was mixed with 30 µl archive kit (Applied Biosystems) master mix containing

- 6 µl reverse transcriptase buffer
- 2.4 µl dNTPs
- 6 µl random primers
- 3 µl Multiscribe reverse transcriptase
- 0.75 µl RNasin
- 11.85 µl water

The solution was incubated at 25^{o}C for 10 minutes followed by 37^{o}C for 2 hours and samples then stored at -20^{o}C.

2.8 Detection of Vα Chains in Vα2$^-$ Cells by PCR

Vα2$^-$ cells were sorted from purified CD4$^+$ T cells from naive B6 mice and naive B6.EF4.1 mice (CD4$^+$, CD44^{lo}, Vα2$^-$), and CD4$^+$ T cells from B6. EF4.1 mice which had been stimulated *in vitro* with env$_{124-138}$ for 3 days (CD4$^+$, CFSE^{lo}, Vα2$^-$). RNA was extracted and cDNA was synthesised as described above. For detection of Vα chains cDNA was subjected to PCR using specific primers for *Vα* TCR chains ((Casanova et al., 1991) see Table 2.4). PCR reactions were carried out in a volume of 20 µl in the suppliers buffer, and contained a final concentration of

- 2 mM $MgCl_2$ (Thermo Scientific)
- 0.2 mM dNTP (G.E. Healthcare)
- 0.4 pmol/µl sense and antisense primer
- 0.1 U *Taq* DNA polymerase (Thermo Scientific)

PCR amplifications were amplified in an Eppendorf mastercycler thermocycler as follows

- 2 min 15 sec denaturation step at 94^{o}C
- 35 cycles of denaturation at 94^{o}C for 15 seconds
- annealing at 55^{o}C for 15 seconds
- extension at 72^{o}C for 30 seconds
- a final extension at 72^{o}C for 2 minutes

PCR products were separated on a 2% agarose gel containing ethidium bromide.

Vα	**Sequence (5'-3')**
Vα1	GCACTGATGTCCATCTTCTC
Vα2	AAAGGGAGAAAAAGCTCTCC
Vα3	AAGTACTATTCCGGAGACCC
Vα4	CAGTATCCCGGAGAAGGTC
Vα5	CAAGAAAGACAAACGACTCTC
Vα6	ATGGCTTTCCTGGCTATTGCC
Vα7	TCTGTAGTCTTCCAGAAATC
Vα8	CAACAAGAGGACCGAGCACC
Vα9	TAGTGACTGTGGTGGATGTC
Vα10	AACGTCGCAGCTCTTTGCAC
Vα11	CCCTGCACATCAGGGATGCC
Vα12	TCTGTTTATCTCTGCTGACC
Vα13.1	ACCTGGAGAGAATCCTAAGC
Vα34S-281	TCCTGGTTGACCAAAAAGAC
VαA10	TGGTTTGAAGGACAGTGGGC
VαBWB	CATTCGCTCAAATGTGAACAG
VαBMA	CAAATGAGAGAGAGAAGCGC
VαBMB	GGAAAATGCAACAGTGGGTC
Vα5T	GACATGACTGGCTTCCTGAAGGCCTTGC
Cαb	ACACAGCAGGTTCTGGGTTC

Table 2.4 Sequences of primers used for detection of Vα TCR chains.

2.9 Assessment of Infection

2.9.1 Spleen index

The spleen was removed from a euthanised mouse and subsequently spleen and mouse were weighed. The following equation was used to calculate the spleen index (SI).

$$SI = \frac{\text{Spleen weight (mg)}}{\text{Mouse weight (g)}}$$

2.9.2 Detection of FV infected cells by flow cytometry

Infected cells were estimated by flow cytometric detection of infected cells using surface staining for the glycosylated product of the viral gag gene (glyco-Gag), using the mAb 34 (mouse IgG2b) for 30 minutes at RT. Cells were washed with FACS buffer and incubated with an anti-mouse IgG2b-FITC secondary reagent (**Table 2.2**) for 20 minutes at RT. Cells were then washed and resuspended in FACS buffer for FACS analysis.

2.9.3 Infected cells in the blood

To reduce numbers of mice used, in some experiments mice were assessed for infection by bleeding. Red blood cells were lysed using BD FACS Lysing Solution (BD Biosciences). FV-infected cells were detected by flow cytometry as described. **Figure 2.2** shows the gating strategy employed throughout to detect infected cells in the blood or spleen cell suspension.

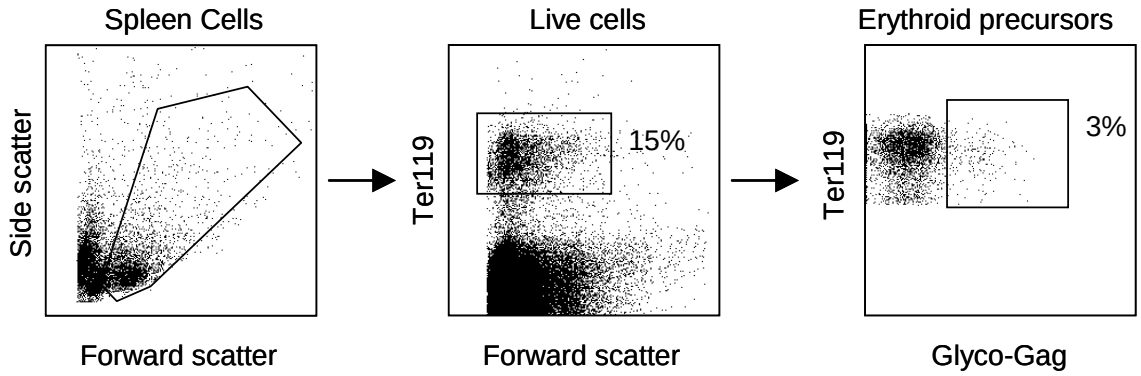

Figure 2.2 Gating strategy for identification of FV infected cells

Flow cytometric example of the gating applied to define the infected (glyco-Gag$^+$) erythroid precursors (Ter119$^+$) cells in the spleen. Cells were stained for surface antibodies and live cells were gated on size and granularity. Ter119$^+$ cells within the live cell population were gated and the percentage is shown. Glyco-gag$^+$ cells (infected cells) are gated and infected cells within the total Ter119$^+$ cell population are shown.

2.9.4 Real-Time quantitative PCR

In order to detect the minimum FV-MuLV copy number detectable by quantitative PCR (Q-PCR), the pFMU3A plasmid (10936 bp) was titrated from 100,000 copies (1.2pg plasmid DNA) in two-fold dilutions to generate a standard curve (Cycle threshold (Ct) value vs F-MuLV *env* copy number). This showed that the sensitivity of the assay was 6 copies of proviral DNA (Ct= 35) (**Figure 2.3**).

The SYBR green PCR Master Mix (Applied Biosystems, Warrington, UK/ Foster City, CA) was used for DNA analysis by real-time Q PCR with a set of primers for Friend *env*:

forward 5'-CTGCGCCAGAGACTGCGACGA-3'

reverse 5'-GACCCGGGGCAGACATAAAAT-3'

Samples for RT-PCR were run on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems) and expression of F-MuLV-DNA was normalised to that of *Ifnα/βr*. The primers used to amplify *Ifnα/βr* were as follows:

forward 5'-AAGATGTGCTGTTCCCTTCCTCTGCTCTGA-3'

reverse 5'-ATTATTAAAAGAAAAGACGAGGCGAAGTGG-3'

600 ng of DNA (corresponding to 100,000 cells) was used per reaction. Ct values were used to calculate number of proviral DNA copies per 100,000 cells from the standard curve.

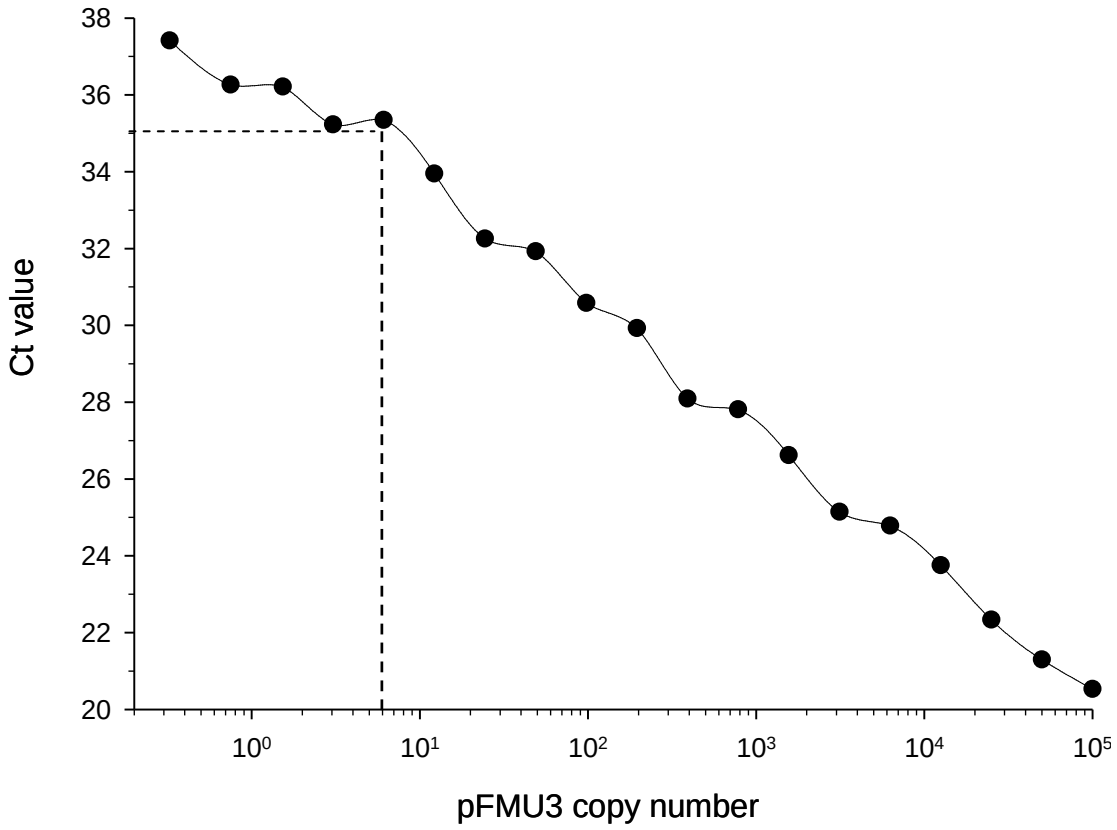

Figure 2.3 Titration of pFMU3 plasmid copy number by Q-PCR

Plasmid pFMU3 was titrated from 100,000 copies to <1 copy. The Ct value for each copy number is shown. A dashed line represents the limit of detection of the assay (Ct value=35, copy number=6).

2.10 Statistical Analysis

Graphs were created and results analysed for statistical significance with SigmaPlot v11.0 (Systat Software Inc., San Jose, CA, USA). Results were analysed with a two-tailed student's t-test or, when data was not normally distributed with the non-parametric two sample independent Wilcoxon signed rank test. p values <0.05 were considered to be statistically significant.

Results and Discussion

Chapter Three

3 Control of FV Infection by $CD4^+$ T cells

3.1 Introduction

Depletion of $CD4^+$ T cells in mice has been shown to have only minor effects on recovery from acute FV infection, while depletion of $CD8^+$ T cells prevented recovery from acute FV (Robertson et al., 1992). Conversely, depletion during the chronic phase of FV infection resulted in reactivation of viral replication, leading to the conclusion that $CD4^+$ T cells are required for control of the chronic phase of FV infection but are not required for control of acute infection (Hasenkrug et al., 1998; Hasenkrug, 1999; Robertson et al., 1992). Furthermore, attempts to induce a protective FV-specific $CD4^+$ T cell response using peptide immunisation or attenuated virus vaccine varied, and were ultimately inconclusive (Dittmer et al., 1999; Miyazawa et al., 1995).

It should be emphasised that these studies were carried out using an FV stock which was potentially contaminated with LDV, resulting in FV/LDV coinfection. LDV infection has profound effects on the immune response to FV, including polyclonal B cell activation and delayed $CD8^+$ T cell responses (Marques et al., 2008; Robertson et al., 2008). These factors may have contributed to inconclusive, or even erroneous results in the above mentioned studies.

Importantly, it has recently been shown that immunocompetent mice have lower levels of FV infection in the spleen than $CD4^+$ T cell-deficient mice after infection with a clean, LDV-free FV stock (Pike et al., 2009). This provides evidence for a role for $CD4^+$ T cells in controlling acute FV infection and suggests that they are required

in the primary immune response against FV, in contrast to studies concluding that the major role of $CD4^+$ T cells in FV infection was during the chronic phase.

This observation, as well as the potential virus contamination of previously used FV stocks lead us to reassess the contribution of $CD4^+$ T cells against FV infection. The EF4.1 TCRβ-transgenic mouse was employed in order to further investigate the role of FV-specific $CD4^+$ T cells during the acute phase of FV infection. Here, the potential protective capacity of FV-specific $CD4^+$ T cells was investigated, and the mechanisms of action by which the presence of $CD4^+$ T cells during acute infection can reduce the level of infection in the spleen were elucidated.

3.2 The Role of $CD4^+$ T Cells against Acute FV Infection

3.2.1 Induction of FV-specific $CD4^+$ T cells

Peptide immunisation with a Th cell epitope was previously found to be sufficient to partially protect mice from FV-induced splenomegaly and erythroleukaemia in the presence of LDV (Miyazawa et al., 1995). In order to test whether immunisation with $env_{124-138}$ peptide was adequate to induce a FV-specific memory $CD4^+$ T cell, 2×10^6 EF4.1 TCRβ-transgenic $CD4^+$ T cells were transferred into mice one day before immunisation with F-MuLV $env_{124-138}$ peptide and adjuvant. Mice were infected with FV 35 days after immunisation, and the percentage of FV infected erythroid precursor cells in the spleen day 7 after infection was assessed with flow cytometry to identify $glyco^-Gag^+Ter119^+$ cells in the spleen, as demonstrated in **Figure 2.2**. A control group was FV-infected in parallel.

Compared to controls, mice which had been immunised 35 days before infection showed significantly lower levels of infected cells in the spleen ($p=0.01$) demonstrating that immunisation with $env_{124-138}$ peptide was adequate to induce a protective FV-specific memory $CD4^+$ T cell response (**Figure 3.1**). This was consistent with previous observations that peptide immunisation to induce a memory $CD4^+$ T cell response is able to protect against FV infection in the presence of LDV coinfection (Miyazawa et al., 1995).

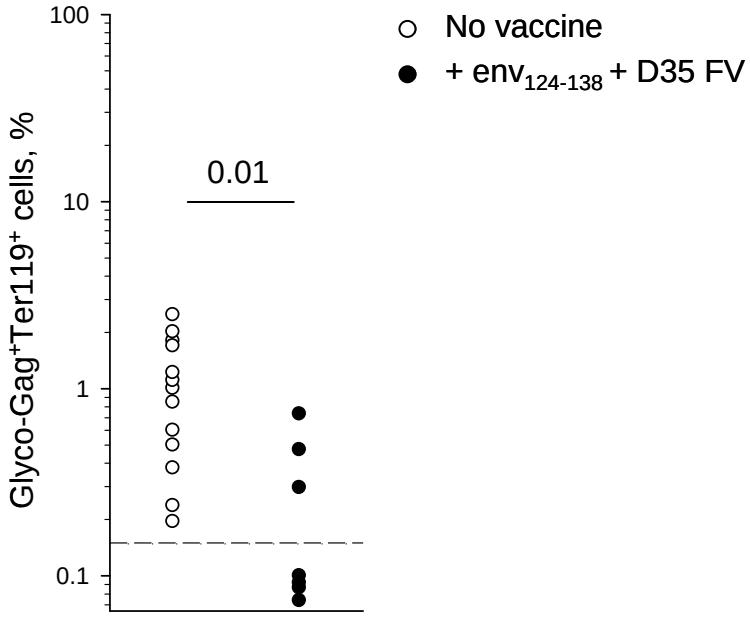

Figure 3.1 Control of FV infection 35 days after adoptive transfer and immunisation with FV env peptide

B6 mice received 2 × 10^6 EF4.1 TCRβ-transgenic $CD4^+$ T cells one day before immunisation with $env_{124\text{-}138}$ and adjuvant. Percentage of glyco-$Gag^+Ter119^+$ cells in the spleen of FV-infected mice and mice FV-infected 35 days after immunisation are shown. Each symbol represents an individual mouse. The dashed line indicates the limit of flow cytometric detection. The number within the graph denotes the *p* value as compared using a two-tailed Wilcoxon-Mann-Whitney test.

3.2.2 The effect of additional FV-specific $CD4^+$ T cells on FV infection

As shown above, peptide immunisation to induce an FV-specific $CD4^+$ T cell response was adequate to reduce the level of FV infection at the peak. However, it is possible that peptide-immunisation induced FV-specific $CD4^+$ T cells reach a different level of differentiation than FV-specific $CD4^+$ T cells responding in a primary response to FV infection would. Thus, the protective effect of naive FV-specific $CD4^+$ T cells on primary FV infection was examined.

To evaluate the contribution of additional $CD4^+$ T cells to protection against FV infection, TCRβ-transgenic $CD4^+$ T cells were adoptively transferred into B6 mice one day before FV infection and the level of infection measured at different time-points. A clear population of $glyco^-Gag^+Ter119^+$ cells (2%) was seen at day 7 after infection of B6 mice which had not received TCRβ-transgenic $CD4^+$ T cells (**Figure 3.2A top**). However, in mice which had received TCRβ-transgenic $CD4^+$ T cells, this population of infected cells was clearly reduced (0.2%) (**Figure 3.2A bottom**).

In B6 mice, infection peaked at day 7 and was resolved by day 21, when the fraction of infected cells in the spleen was below the level of detection by flow cytometry. Adoptive transfer of 10^6 TCRβ-transgenic $CD4^+$ T cells was able to significantly reduce the percentage of infected erythroid precursors in the spleen at the peak of infection in B6 mice ($p=0.034$) (**Figure 3.2B**).

In order to investigate whether transfer of TCRβ-transgenic $CD4^+$ T cells was able to significantly reduce the level of FV infection in mice with the *Fv2* susceptibility allele, this experiment was repeated in B6.A-$Fv2^s$ mice, which have a higher percentage of infected cells at the peak of infection but, like B6 mice, resolve the acute phase of infection by day 21. Transfer of TCRβ-transgenic $CD4^+$ T cells one day before FV infection of B6.A-$Fv2^s$ mice was once more able to protect against FV, and significantly decreased the proportion of infected cells in the spleen at the peak of infection, in this case by ~10-fold ($p<0.00001$) (**Figure 3.2C**). Additionally, this protective effect was shown to be dose-dependent, with the anti-retroviral effect further improved when mice received increased numbers of TCRβ-transgenic $CD4^+$ T cells. This effect was seen in both B6 mice ($p=0.001$) (**Figure 3.3A**) and B6.A-$Fv2^s$ mice ($p=0.0007$) (**Figure 3.3B**).

These data therefore showed that elevated numbers of FV-specific $CD4^+$ T cells were sufficient to significantly reduce infection in both B6 wild-type mice, and mice with a more severe disease due to the presence of the *Fv2* susceptibility allele. Moreover, the protective effect seen was proportional to the number of additional FV-specific $CD4^+$ T cells transferred. This demonstrated a previously unappreciated role for $CD4^+$ T cells in the primary immune response to FV infection, and in controlling the spread of FV infection.

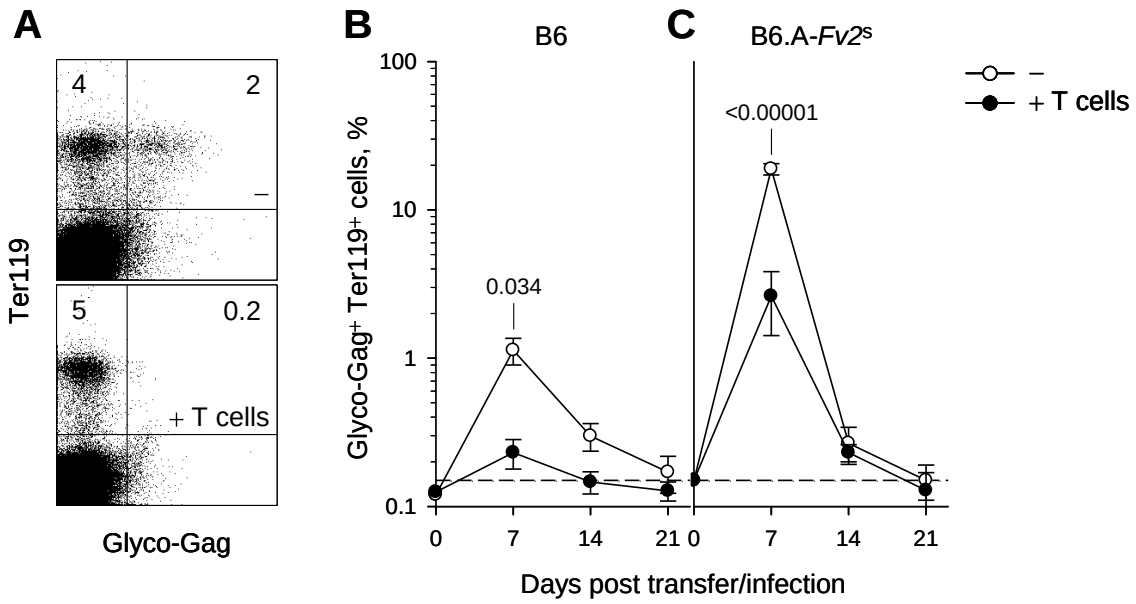

Figure 3.2 Control of FV infection after adoptive transfer of additional FV-specific CD4$^+$ T cells

(A) B6 control mice (-) and B6 mice which received 1 × 10^6 EF4.1 TCRβ-transgenic CD4$^+$ T cells 1 day before infection (+ T cells), were infected with FV and the percentage of FV-infected (glyco-Gag$^+$) Ter119$^+$ cells in the spleen, 7 days post infection, is shown. Numbers within the quadrants denote the percentage of positive cells.

(B-C) Percentage of glyco-Gag$^+$Ter119$^+$ cells in the spleen of B6 (B) or B6.A-*Fv2*^{s} (C) mice after FV infection, with (+ T cells) or without (-) adoptive transfer of 1 × 10^6 TCRβ-transgenic CD4$^+$ T cells. Values are the means (± the SEM) of 4-12 mice per group per time point. The dashed line indicates the limit of flow cytometric detection. Numbers within the graph denote the *p* values as compared using a two-tailed student's t-test.

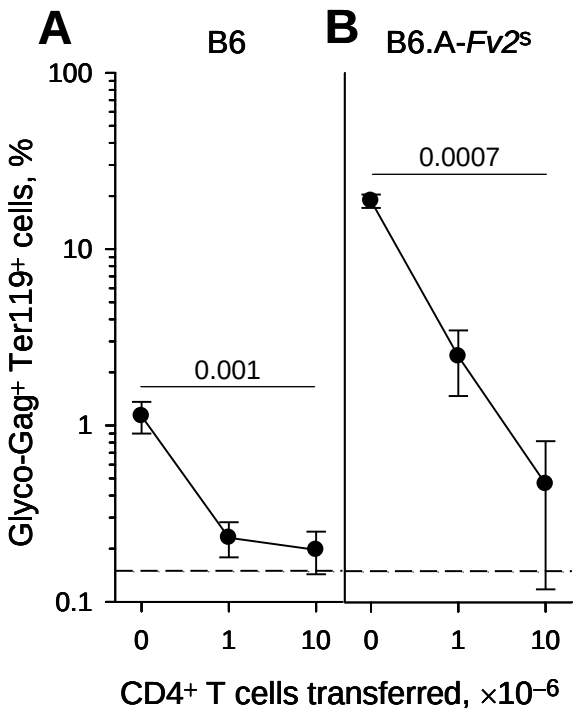

Figure 3.3 Dose-dependent reduction of FV infection by increased number of additional FV-specific CD4$^+$ T cells

(A-B) Percentage of glyco-Gag$^+$Ter119$^+$ cells in the spleen of B6 (A) or B6.A-*Fv2*^{s} mice (B), 7 days after FV infection after adoptive transfer of titrated numbers of EF4.1 TCRβ-transgenic CD4$^+$ T cells 1 day before infection. Values are the mean (± the SEM) of 3-10 mice per group per CD4$^+$ T cell inoculum from 1-3 separate experiments (A), or 2-12 mice from 1-4 separate experiments (B). The dashed line indicates the limit of flow cytometric detection. Numbers within the graph denote the *p* as compared using a two-tailed student's t-test.

3.3 Mechanisms of Anti-Retroviral $CD4^+$ T Cells in FV Infection

$CD4^+$ T cells are conventionally helpers of the immune system, providing immunological help to B cells and $CD8^+$ T cells to enhance antibody production and cytotoxic killing of virally infected cells, respectively. However, $CD4^+$ T cells have also been shown to possess direct anti-viral activity. This anti-viral effect can occur via production of anti-viral cytokines such as IFN-γ, or direct killing of virally-infected cells (Jellison et al., 2005). With this in mind, we sought to discover whether anti-retroviral $CD4^+$ T cells in FV infection were functioning via provision of immunological help to other adaptive lymphocytes, or via an independent anti-viral mechanism.

3.3.1 Effect of additional FV-specific $CD4^+$ T cells on FV nAb titre

Induction of virus-nAb is a key effector mechanism by which B cells can protect against and control viral infection. To ascertain whether FV-specific $CD4^+$ T cells were providing immunological help to B cells, FV nAb titre was measured during infection of mice which had received EF4.1 TCRβ-transgenic $CD4^+$ T cells one day before FV infection.

In both the control group and the group which had received TCRβ-transgenic $CD4^+$ T cells, FV nAb was only induced to a level detectable by the assay used at day 14 post infection and, while the FV nAb titre continued to increase, by day 21 post infection no increased production of FV nAb in mice which had received TCRβ-transgenic $CD4^+$ T cells was observed compared to control mice (**Figure 3.4**).

While these experiments showed that additional FV-specific $CD4^+$ T cells did not increase levels of nAb against the virus, FV nAb was not detected until day 14 after infection while $CD4^+$ T cell-mediated protection against FV infection in B6 mice occurred at day 7. This provided further evidence suggesting that control of FV by additional FV-specific $CD4^+$ T cells was not facilitated via increased FV nAb production by B cells.

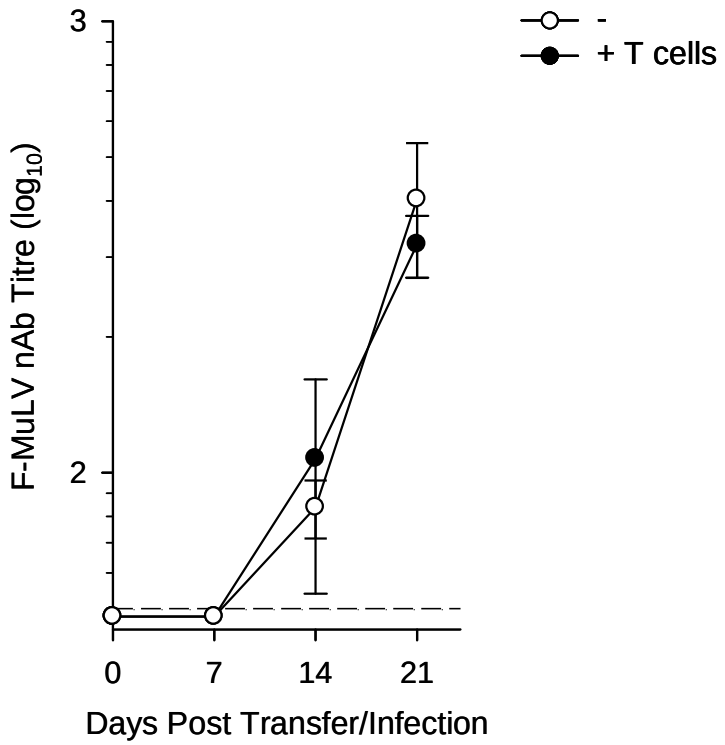

Figure 3.4 Effect of additional FV-specific $CD4^+$ T cells on FV nAb titre

The FV nAb titre of B6 mice with (+ T cells) or without (-) adoptive transfer of 1×10^6 TCRβ-transgenic $CD4^+$ T cells one day before FV infection is shown. Values are the mean (± the SEM) of 6-9 mice per group per time point from 2 experiments. The dashed line indicates the limit of detection of the FV nAb assay.

3.3.2 Effect of FV-specific $CD4^+$ T cells on primary FV infection in lymphopenic mice

The above data indicate that additional FV-specific $CD4^+$ T cells are not exerting their anti-retroviral role via induction of nAb production by B cells. However, they may still be helping B cells in other ways, for example inducing opsonising antibody production by B cells, resulting in killing of infected cells. Additionally, anti-retroviral $CD4^+$ T cells could exert this effect via helping $CD8^+$ T cells to kill virally-infected cells. In order to discount the possibility that $CD4^+$ T cells were exerting the observed anti-viral effect via help to other adaptive lymphocytes, the effect of transfer of EF4.1 TCRβ-transgenic $CD4^+$ T cells on FV-infection in B6.A-*Fv2*^{s} *Rag1*$^{-/-}$ mice was investigated. B6.A-*Fv2*^{s} *Rag1*$^{-/-}$ mice, in addition to the *Fv2*^{s} allele, have no endogenous B or T lymphocytes and suffer from massive splenomegaly, eventually succumbing to disease. Any protection seen in this model after transfer of TCRβ-transgenic $CD4^+$ T cells would show that this protective effect was independent of B cells and $CD8^+$ T cells, and so was not via provision of help to these cells by $CD4^+$ T cells.

To assess the effect of EF4.1 TCRβ-transgenic $CD4^+$ T cells on initial FV infection in B6.A-*Fv2*^{s} *Rag1*$^{-/-}$ mice, the percentage of infected erythroid precursor cells in the blood was compared to the percentage of $CD4^+$ T cells in the blood at day 7 post infection. There was a direct inverse correlation between the two (r2=0.909), where mice with a higher percentage of $CD4^+$ T cells in the blood had a lower percentage of $glyco^-Gag^+Ter119^+$ cells in the blood, showing that the presence of FV-specific $CD4^+$

T cells was adequate to reduce the level of FV infection in the blood during primary infection in the absence of $CD8^+$ T cells and B cells (**Figure 3.5**).

To further investigate this B cell- and $CD8^+$ T cell-independent role of $CD4^+$ T cells against FV infection, the effect of titrated numbers of TCRβ-transgenic $CD4^+$ T cells on splenomegaly in B6.A-*Fv2*^{s} *Rag1*$^{-/-}$ was assessed. Mice which had not received TCRβ-transgenic $CD4^+$ T cells began to develop splenomegaly by day 10, and all mice in this group had developed large spleens by day 14 post infection. However, mice which had received TCRβ-transgenic $CD4^+$ T cells did not develop splenomegaly during this time, and at day 14 had significantly smaller spleens than control mice ($p=0.00001$). Furthermore, as few as 10^5 TCRβ-transgenic $CD4^+$ T cells were sufficient to protect mice against splenomegaly until day 21 post infection, while transfer of 10^6 TCRβ-transgenic $CD4^+$ T cells was able to protect mice beyond day 21 post infection. Although protection against splenomegaly was not maintained beyond day 28 after infection, transfer of TCRβ-transgenic $CD4^+$ T cells resulted in a significant delay in the onset of splenomegaly in mice (**Figure 3.6**).

These data demonstrated that FV-specific $CD4^+$ T cells were able to protect against FV infection in the absence of endogenous adaptive lymphocytes in mice with genetic susceptibility to FV disease. Although transfer of EF4.1 TCRβ-transgenic $CD4^+$ T cells was unable to prevent eventual onset of splenomegaly during the later stage of infection in B6.A-*Fv2*^{s} *Rag1*$^{-/-}$ mice, it was sufficient to control the initial infection and significantly delay onset of FV-induced disease via a mechanism which did not involve provision of immunological help to B cells or $CD8^+$ T cells.

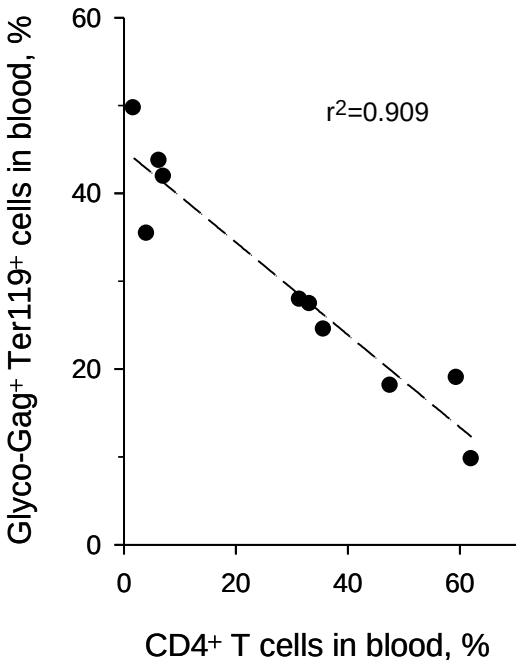

Figure 3.5 CD8$^+$ T cell- and B cell-independent reduction of FV infection by FV-specific CD4$^+$ T cells

Correlation between the percentage of FV-infected (glyco-Gag$^+$) Ter119$^+$ cells and the percentage of donor CD4$^+$ T cells in the blood, 7 days after FV infection of lymphopenic B6.A-*Fv2^s Rag1$^{-/-}$* mice which received titrated numbers of EF4.1 TCRβ-transgenic CD4$^+$ T cells. Each symbol represents an individual mouse. The dashed line represents the line of correlation (r^2=0.909).

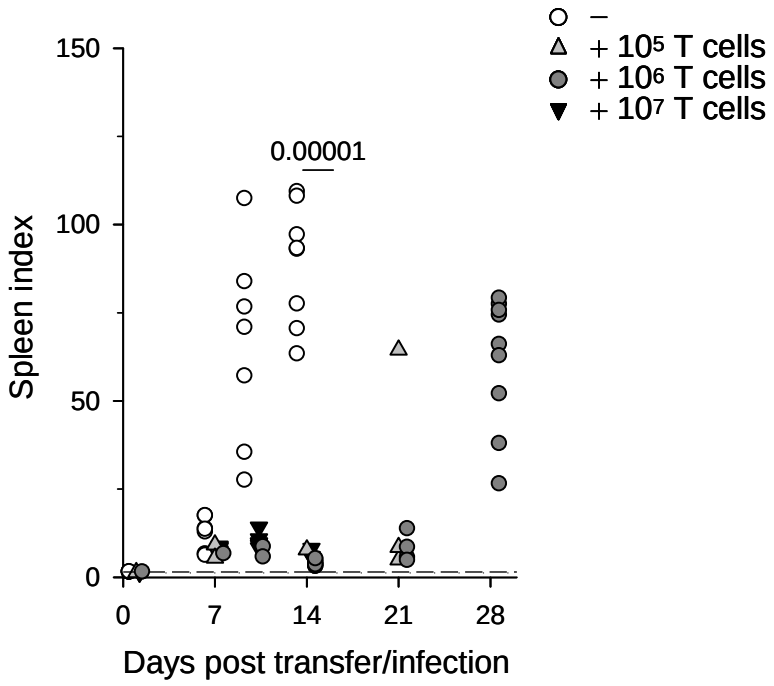

Figure 3.6 CD8$^+$ T cell- and B cell-independent protection against FV-induced splenomegaly by FV-specific CD4$^+$ T cells

Spleen index after FV infection of B6.A-*Fv2^s Rag1$^{-/-}$* mice with (+ T cells), or without (-) adoptive transfer of indicated numbers of EF4.1 TCRβ-transgenic CD4$^+$ T cells. Each symbol represents an individual mouse. The dashed line indicates the spleen index of uninfected mice. Numbers within the graph denote the *p* values as compared using a two-tailed Wilcoxon-Mann-Whitney test.

3.4 Cytokine Production by FV-specific $CD4^+$ T Cells

3.4.1 Lineage-specific cytokine production by FV-specific $CD4^+$ T cells

$CD4^+$ T cells can be characterised and divided into Th cell subsets depending on their cytokine production. Intra-cellular cytokine staining of FV-specific $CD4^+$ T cells at day 7 post infection was carried out to identify which T helper cell subset was dominating the anti-retroviral protective response against FV infection. This revealed that the responding FV-specific $CD4^+$ T cell population was comprised of IL-2 single producers and IFN-γ single producers, as well as IL-2/IFN-γ double producer $CD4^+$ T cells (**Figure 3.7A**). In contrast, there was negligible production of IL-17A by the FV-specific $CD4^+$ T cell population, either alone or in concert with IFN-γ, discounting the possibility of a Th17 cell phenotype (**Figure 3.7B**). These data indicated that the FV-specific $CD4^+$ T cell population had an inclination towards a Th1 $CD4^+$ T cell subset, with IL-2 and IFN-γ representing signature Th1 cytokines.

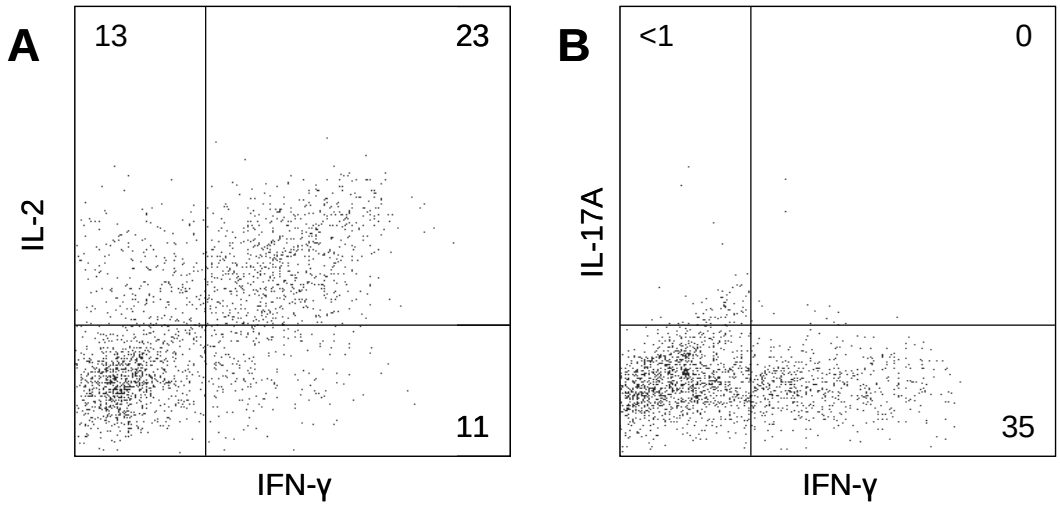

Figure 3.7 Cytokine production by FV-specific CD4$^+$ T cells

2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice (CD45.2$^+$) 1 day before FV infection. Numbers within the quadrants denote the percentages of positive cells. Production of IFN-γ and IL-2 (A) and IFN-γ and IL-17 (B) by FV-specific donor CD4$^+$ T cells is shown.

3.4.2 IFN-γ in $CD4^+$ T cell-mediated control of primary FV infection

$CD4^+$ T cells have previously been shown to be able to inhibit viral replication via production of the anti-viral cytokine IFN-γ (Christensen et al., 1999; Davis et al., 2008). In order to determine whether protective $CD4^+$ T cells in the FV model were acting via production of IFN-γ, the ability of additional FV-specific $CD4^+$ T cells to reduce FV infection in B6 mice deficient in the IFN-γ receptor (B6-*Ifngr1*$^{-/-}$ mice) was investigated.

Adoptive transfer and infection experiments were repeated in B6-*Ifngr1*$^{-/-}$ mice and B6 controls. Transfer of EF4.1 TCRβ-transgenic $CD4^+$ T cells was able to reduce infection at the peak compared to mice which had not received $CD4^+$ T cells, as shown above ($p=0.0001$). Notably, transfer of EF4.1 TCRβ-transgenic $CD4^+$ T cells before FV infection of B6-*Ifngr1*$^{-/-}$ mice was able to reduce the percentage of infected erythroid precursor cells in the spleen compared to mice which did not receive $CD4^+$ T cells, despite the inability of endogenous cells to respond to IFN-γ ($p=0.004$) (**Figure 3.8**). This demonstrates that anti-retroviral FV-specific $CD4^+$ T cells in the FV model could exert their protective effect independently of IFN-γ. Together, these results showed that anti-retroviral FV-specific $CD4^+$ T cells were able to control FV infection via a $CD8^+$ T cell- and B cell-independent mechanism, and that this FV-specific $CD4^+$ T cell-mediated anti-retroviral effect was unlikely to be mediated by production of IFN-γ, but was instead operating through an unconventional anti-viral mechanism of $CD4^+$ T cells.

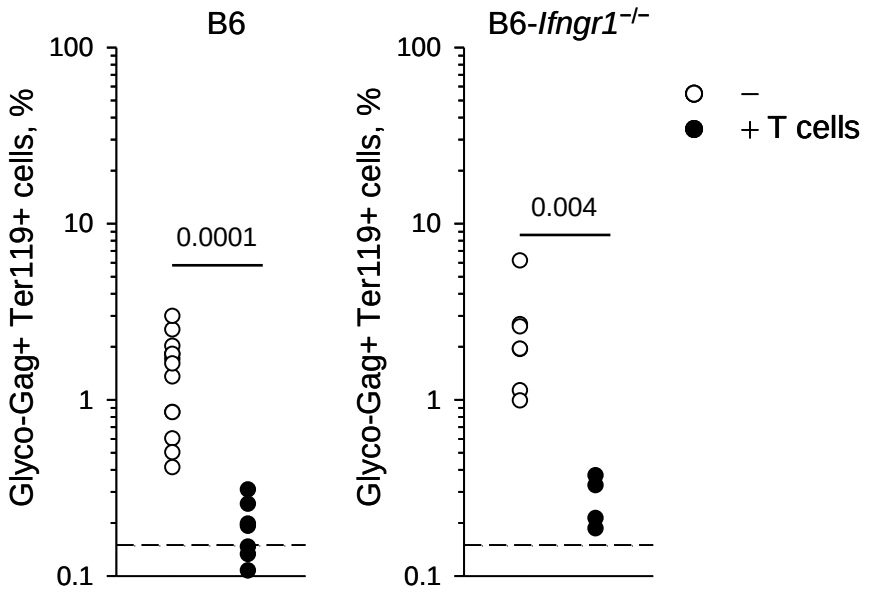

Figure 3.8 Control of FV infection by FV-specific TCRβ-transgenic CD4$^+$ T cells in IFN-γR deficient recipients

Percentage of glyco-Gag$^+$Ter119$^+$ cells in the spleen of B6 or B6-*Ifngr1*$^{-/-}$ mice, 7 days after FV infection, with (+ T cells) or without (-) adoptive transfer of 1×10^6 EF4.1 TCRβ-transgenic CD4$^+$ T cells 1 day before infection. Each symbol represents an individual mouse. The dashed line indicates the limit of flow cytometric detection. Numbers within the graph denote the *p* values as compared using a two-tailed Wilcoxon-Mann-Whitney test.

3.5 Discussion

3.5.1 Peptide immunisation and protection against FV

Mice infected with FV 35 days after cell transfer and immunisation with FV env peptide were shown to have a reduced level of infection in the spleen, with protection comparable to that observed in mice infected one day after cell transfer. This suggests that peptide immunisation is able to induce expansion of FV-specific $CD4^+$ T cells to a level where they are still present in numbers sufficient to reduce FV infection by day 35 after infection. Previous studies revealed a protective effect of peptide immunisation which induced a protective FV-specific $CD4^+$ T cell response. However, one of these peptides (C-terminal) was shown to have an Ab binding epitope that, although it did not induce nAb, may have affected the reliability of the results obtained (Miyazawa et al., 1995). Although this previous study was carried out using a potentially LDV-contaminated FV stock, the current results presented here confirm that induction of FV-specific $CD4^+$ T cells by peptide immunisation is able to induce a protective $CD4^+$ T cell response against FV in mice infected with FV alone as well as in FV/LDV co-infection. However, the phenotype of the FV-specific $CD4^+$ T cells induced is not defined, and so it is not known whether peptide immunisation is inducing a protective response by increasing the number of FV-specific $CD4^+$ T cells, or by inducing a memory $CD4^+$ T cell population which is able to mount a more rapid and effective response against FV infection.

3.5.2 $CD4^+$ T cell-mediated control of acute FV infection

The essential role of $CD4^+$ T cells in the immune response to pathogens is evident in both primary and secondary immunodeficiency in humans and in animal models. With regards to FV, $CD4^+$ T cells have been shown to make a major contribution to controlling the chronic phase of infection in immunocompetent mice (Hasenkrug et al., 1998; Robertson et al., 1992). It has also been previously shown that adoptive transfer of whole immune spleen cells from mice immunised with an attenuated F-MuLV is able to reduce FV infection (Dittmer et al., 1998). However, when adaptive lymphocytes were divided into the 3 major adaptive lineages, adoptive transfer of $CD8^+$ T cells was able to reduce FV infection in naive mice, but transfer of $CD4^+$ T cells from these immune mice was not (Dittmer et al., 1999). This study concluded that $CD4^+$ T cells alone were not adequate for protection against FV. However, these previous studies and others were carried out using an FV stock likely to be contaminated with LDV. As discussed, coinfection of LDV with FV has profound effects on FV pathogenesis and induction of the FV-specific immune response. Infection of $CD4^+$ T cell-deficient mice with clean, uncontaminated FV stock has demonstrated a clear role for $CD4^+$ T cells in controlling the acute phase of FV infection (Pike et al., 2009). Here, the effect of adoptive transfer of EF4.1 TCRβ-transgenic $CD4^+$ T cells on FV infection using clean FV stock was assessed.

The results here show that, in contrast to studies using an LDV-contaminated FV stock, additional FV-specific $CD4^+$ T cells are able to contribute to protection against FV infection and in fact, adoptive transfer of these transgenic $CD4^+$ T cells significantly reduces the percentage of FV-infected cells in the spleen during the acute

phase of infection. This significant reduction of infection is observed in both immunocompetent *Fv2*^{r} B6 mice, and mice with susceptibility at the *Fv2* locus. These data show for the first time that the presence of FV-specific CD4^{+} T cells during acute infection contributes to control of FV infection and thus that CD4^{+} T cells have an anti-retroviral role during the acute phase of FV infection.

In experiments using LDV-contaminated FV stock, increasing the number of whole immune spleen cells in adoptive transfer experiments improved protection against FV infection (Dittmer et al., 1998). Furthermore, transfer of immune CD8^{+} T cells was able to protect against FV infection, while transfer of immune CD4^{+} T cells was not (Dittmer et al., 1999). Increasing the number of immune CD8^{+} T cells transferred also resulted in a further improvement in protection, no protection was observed when the number of immune CD4^{+} T cells transferred was increased (Dittmer and Hasenkrug, 2000). Here it is shown that transfer of increased numbers of EF4.1 TCRβ-transgenic CD4^{+} T cells in the absence of FV/LDV co-infection results in a significant reduction of FV-infected cells in the spleen during the acute phase of infection, demonstrating a role for CD4^{+} T cells against FV infection which was previously unrecognised. Furthermore, CD4^{+} T cell-mediated protection occurred in a dose-dependent manner, with protection improving upon adoptive transfer of increased numbers of EF4.1 CD4^{+} T cells, showing that protection is proportional to CD4^{+} T cell number, and contradicting further experiments.

During FV/LDV-coinfection, the peak of FV infection in the spleen is observed at day 14, rather than day 7 as seen during FV infection alone, and although additional FV-

specific $CD4^+$ T cells are still able to reduce FV infection at the peak in FV/LDV co-infected mice, more $CD4^+$ T cells are necessary to reduce FV infection when LDV is also present (Pike et al., 2009). This may explain the previous observation that increasing the number of immune $CD4^+$ T cells had no effect on FV infection in studies which possibly used an LDV-contaminated stock. It is possible that the attenuated virus vaccine used to induce immune mice from which these FV-specific $CD4^+$ T cells were obtained may have induced adequate numbers to control FV infection alone, but not to the magnitude where they were able to control FV/LDV co-infection due to the increased level of infection.

3.5.3 An independent role for $CD4^+$ T cells against FV infection

Further to the observation that additional FV-specific $CD4^+$ T cells were able to reduce FV infection during the acute phase, the mechanism by which this anti-viral effect was mediated was elucidated. Experiments were carried out in order to assess whether FV-specific $CD4^+$ T cells were controlling FV infection via immunological help or via a direct effect against FV infected cells.

FV nAb has been shown to play an important role against FV infection, and administration of FV nAb in the absence of B cells results in a reduction of FV infection (Messer et al., 2004). Furthermore, mice with FV/LDV coinfection suffer a more severe infection, partially due to delayed onset of FV nAb production caused by LDV (Marques et al., 2008). With this in mind, the effect of additional FV-specific $CD4^+$ T cells on FV nAb titre during FV infection was studied. It was observed that adoptive transfer of TCRβ-transgenic $CD4^+$ T cells before FV infection did not lead to

increase in FV nAb titre compared to control mice. Additionally, $CD4^+$ T cell-mediated control of FV infection was observed at day 7 after infection, while FV nAb was not induced to a detectable level until day 14 after infection. This showed that additional FV-specific $CD4^+$ T cells in this model are not reducing FV infection via provision of immunological help to B cells to increase the level of production of nAb against the virus.

To determine whether $CD4^+$ T cells were able to control FV infection in the absence of other adaptive lymphocytes, and hence further demonstrating that their anti-retroviral effect was not via immunological help, adoptive transfer and infection experiments were repeated in *Fv2* susceptible mice with no endogenous T cells or B cells. Adoptive transfer of FV-specific $CD4^+$ T cells was able to reduce levels of infection in the blood at day 7 after infection, with an inverse correlation observed between the percentage of $CD4^+$ T cells in the blood and the percentage of infected erythroid precursors in the blood. Additionally, while control mice developed splenomegaly by day 10 after infection, mice which had received EF4.1 TCRβ-transgenic $CD4^+$ T cells maintained significantly smaller spleens after day 10. Notably, although protection was not maintained past day 28 after infection, there was a significant delay in the onset of splenomegaly in these mice, providing evidence that FV-specific $CD4^+$ T cells were able to control FV infection and the spread of FV in the absence of both B cells and $CD8^+$ T cells. These data showed that the observed role of $CD4^+$ T cells against FV infection is both B cell- and $CD8^+$ T cell-independent and that these cells were not exerting their anti-retroviral effect via immunological help to other adaptive immune cells, and instead may be exerting a direct anti-retroviral activity against FV infection.

3.5.4 Cytokine production in the control of FV infection

An alternative mechanism to providing help to other adaptive lymphocytes, by which FV-specific $CD4^+$ T cells may have been controlling FV infection, is production of anti-viral cytokines such as interferons, which are able to exert direct anti-viral activity. $CD4^+$ T cell responses to intracellular infections, including viral infections, are typically mediated by the IFN-γ-producing Th1 $CD4^+$ T cell subset (Zhu et al., 2010). Intracellular cytokine staining of the responding EF4.1 TCRβ-transgenic $CD4^+$ T cell population demonstrated that the FV-specific $CD4^+$ T cell population contained both IFN-γ and IL-2 single producers, as well as IFN-γ/IL-2 double producers, and hence exhibited characteristics of a Th1 phenotype, as predicted. However, very few cells in the FV-specific $CD4^+$ T cell population produced IL-17, and hence this population did not contain cells of the Th17 subset, which correlates with the observation that Th17 cells are associated mainly with parasite infections and autoimmunity.

$CD4^+$ T cells have previously been shown to have direct anti-viral effects via production of IFN-γ hepatitis C virus (HCV) infection and γ-herpesvirus infection (Christensen et al., 1999; Davis et al., 2008). However, efforts to establish the role of $CD4^+$ T cell-derived IFN-γ in the control of FV infection have so far been inconclusive. Previously in FV, while one study has shown a direct inhibition of viral replication by IFN-γ (Iwashiro et al., 2001b), another suggested a contrasting function, with the absence of IFN-γ resulting in decreased infection and an increased nAb titre (Stromnes et al., 2002). A further study suggested that IFN-γ may be detrimental during certain stages of FV infection, but beneficial during others (Stromnes et al., 2002).

With the observation of IFN-γ production by FV-specific $CD4^+$ T cells in mind, the potential role of $CD4^+$ T cell-derived IFN-γ in control of acute FV infection was investigated by infection of mice deficient in the IFN-γ receptor. It was observed that adoptive transfer of additional FV-specific $CD4^+$ T cells in these mice was still able to reduce FV infection in spite of the inability of endogenous cells to respond to IFN-γ. This showed that the protective effect of FV-specific $CD4^+$ T cells was not mediated via a direct effect of IFN-γ on infected cells, or by activation of endogenous innate immune cells by IFN-γ. These observations suggest that IFN-γ does not have a role in $CD4^+$ T cell-mediated control of acute FV infection. However, it is possible that it may be important during the control of chronic infection, and this cannot be ruled out by these experiments.

3.5.5 Potential mechanisms of $CD4^+$ T cell control of FV infection

The data presented here demonstrate a B cell-, $CD8^+$ T cell-, and IFN-γ-independent role for $CD4^+$ T cells against FV infection, indicating that the anti-retroviral effect of FV-specific $CD4^+$ T cells occurs via an alternative mechanism. The observation that FV-specific $CD4^+$ T cells have a direct role in controlling acute FV infection supports previous results showing that $CD4^+$ T cells can have direct cytotoxic effects against FV, as well as during other viral infections.

Direct killing of infected cells by $CD4^+$ T cells has been observed previously in FV infection, and although erythroid precursor cells, the major target of FV infection, do not express MHC class II and so cannot be recognised by $CD4^+$ T cells directly, T cell activation was sufficient to induce $CD4^+$ T cell-mediated cytolysis in this previous study (Iwashiro et al., 2001b). Mice deficient in the Fas/FasL pathway were able to control acute, but not chronic infection (Zelinskyy et al., 2004). This is therefore unlikely to be the mechanism by which $CD4^+$ T cells reduce acute infection, but could be the means by which they contribute to the control of persistent FV infection.

The observation that mice deficient in perforin and/or granzymes are less protected against FV infection has been attributed to compromised $CD8^+$ T cell cytotoxicity (Zelinskyy et al., 2004). As $CD8^+$ T cells have been shown to be deficient in these cytolytic molecules during chronic FV infection (Zelinskyy et al., 2005) this may explain why depletion of $CD8^+$ T cells during chronic infection did not affect viral load, and did not result in reactivation of disease. Potentially, cytotoxic $CD4^+$ T cells are taking over the role of $CD8^+$ T cell-mediated killing of FV-infected cells during

chronic FV infection. Hence, elevated levels of FV infection in perforin/granzyme-deficient mice may have been due to compromised cytotoxic activity of $CD4^+$ T cells rather than $CD8^+$ T cells. By using gene microarrays, upregulation of perforin and granzyme B transcription has been observed in FV-specific $CD4^+$ T cells. In contrast, no upregulation of FasL was observed, suggesting that a direct cytotoxic action of FV-specific $CD4^+$ T cells is more likely to be mediated by perforin and granzymes than the Fas/FasL pathway (Ploquin M., personal communication).

IFN-γ has also been shown to enhance $CD4^+$ T cell killing (Iwashiro et al., 2001b), and this may contribute towards control of acute infection as despite IFN-γR deficient mice still being protected against FV infection to the same extent as those who can respond to IFN-γ, TCRβ-transgenic $CD4^+$ T cells still have a functional IFN-γ receptor and hence endogenously produced IFN-γ, or FV-specific $CD4^+$ T cell derived IFN-γ in an autocrine manner, may increase potential cytotoxic ability of FV-specific $CD4^+$ T cells and hence contribute towards control of acute infection.

$CD4^+$ T cells may alternatively be reducing FV infection via an indirect mechanism, for example, production of cytokines to induce innate immune cells to phagocytose infected cells, or increase activity of NK cells. Importantly, NK cells have been demonstrated to be a major cytotoxic cell type mediating protection against FV infection after peptide immunisation with a $CD4^+$ T cell epitope (Iwanami et al., 2001). As protection against acute infection and delayed splenomegaly by FV-specific $CD4^+$ T cells is observed in mice which have no endogenous adaptive immune response, this suggests that the innate immune system may contribute towards this. In

conclusion, the potential mechanisms by which FV-specific $CD4^+$ T cells are exerting this novel anti-retroviral effect are diverse, and may involve complex networks involving cytokines and innate immune cells.

These data have demonstrated a clear role for $CD4^+$ T cells in the control of acute retroviral infection. This anti-retroviral viral effect is mediated via an unconventional $CD4^+$ T cell mechanism, and may provide further evidence for the importance of cytotoxic $CD4^+$ T cells in control of retroviral infections. These data are also in agreement with studies showing the importance of $CD4^+$ T cells during HIV infection (Rosenberg et al., 1997). Furthermore, the absence of conventional $CD4^+$ T cell mechanisms supports the role of cytotoxic $CD4^+$ T cells during HIV infection (Norris et al., 2001; Norris et al., 2004). These results therefore suggest that vaccine approaches targeting induction of HIV-specific $CD4^+$ T cells provide candidates for further HIV vaccine design. The cause and effect relationship between $CD4^+$ T cell count and HIV viraemia is not defined (Jansen et al., 2006; Lichterfeld et al., 2005), although LTNP's have been shown to have highly functional memory $CD4^+$ T cells (Jansen et al., 2006; Potter et al., 2007). The importance of inducing and maintaining a strong $CD4^+$ T cell response in order to protect against infection by HIV must therefore be further examined.

Chapter Four

4 FV-specific $CD4^+$ T cell Kinetics

4.1 Introduction

$CD4^+$ T cells from EF4.1 TCRβ-transgenic mice have been shown to have an elevated frequency of FV-specific cells compared to those from wild-type mouse strains, with 4% of cells in polyclonal EF4.1 $CD4^+$ T cell population responding to $env_{122-141}$ *in vitro* (Antunes et al., 2008).

Furthermore, it is shown here that additional FV-specific $CD4^+$ T cells are able to reduce the level of FV infection during the acute phase of infection in immunocompetent mice, and delay splenomegaly by a B cell-, $CD8^+$ T cell- or IFN-γ-independent mechanism. To further assess the FV-specific $CD4^+$ T cell response to FV infection, donor $CD4^+$ T cells were identified. The kinetics of FV-specific $CD4^+$ T cells in response to *in vivo* FV infection were studied using an adoptive transfer model. The effect of EF4.1 $CD4^+$ T cell precursor number and antigen dose on expansion and contraction of FV-specific $CD4^+$ T cells was examined.

In response to stimulation, $CD4^+$ T cells proliferate in an antigen-specific manner, and subsequently contract, with ~10% remaining and entering the memory cell pool. In previous studies, it was not possible to identify and quantify the FV-specific $CD4^+$ T cell response, and hence was not possible to study the expansion and contraction of FV-specific $CD4^+$ T cells in response to either FV infection or vaccination techniques. In order to overcome this problem, the B6.EF4.1 TCRβ-transgenic mouse has been developed, and is used in adoptive transfer experiments to elucidate the kinetics of the $CD4^+$ T cell.

4.2 Expansion of EF4.1 TCRβ-transgenic CD4$^+$ T Cells in Response to *in vivo* FV Infection of Immunocompetent Mice

4.2.1 Effect of precursor number on kinetics of FV-specific CD4$^+$ T cells

In order to establish the kinetics of FV-specific CD4$^+$ T cells in response to FV infection, titrated numbers of EF4.1 TCRβ-transgenic CD4$^+$ T cells (10^5, 10^6 or 10^7 cells) were transferred into B6 mice one day before FV infection. Mice were killed at day 1 or 7 post infection and the number of FV-specific donor CD4$^+$ T cells engrafted in the spleen at these time points was quantified with flow cytometry as described (**Figure 2.1**).

In mice that received TCRβ-transgenic CD4$^+$ T cells, but were not infected with FV, there was a negligible percentage of cells expressing CD44 in the donor CD4$^+$ T cell population. However, in mice that had received TCRβ-transgenic CD4$^+$ T cells and were FV-infected, there was a marked expansion of CD44^{hi} donor cells, to comprise ~3% of the entire CD4$^+$ T cell population. This demonstrated that donor TCRβ-transgenic CD4$^+$ T cells expanded and proliferated in response to FV infection (**Figure 4.1A**).

The number of FV-specific donor CD4$^+$ T cells recovered at day 1 after infection was directly proportional to cell expansion of FV-specific donor CD4$^+$ T cells at day 7 post infection (**Figure 4.1B**). The number of FV-specific cells in mice which had received TCRβ-transgenic CD4$^+$ T cells but had not been FV-infected is also shown, and, in the absence of FV infection, no expansion of FV-specific donor CD4$^+$ T cells was observed. Furthermore, the level of expansion of FV-specific CD4$^+$ T cells did

not begin to plateau even when 10^7 TCRβ-transgenic $CD4^+$ T cells were transferred into mice, indicating that the maximum response had not been reached and that upon transfer of larger numbers of TCRβ-transgenic $CD4^+$ T cells, further expansion and hence a stronger FV-specific $CD4^+$ T cell response could potentially be achieved.

Adoptive transfer studies of transgenic $CD4^+$ T cells in the LCMV mouse model have shown that when mice received higher numbers of transgenic $CD4^+$ T cells, virus-specific cells were lost at a more rapid rate (Whitmire et al., 2008). In order to study the effect of precursor number in the continued FV-specific $CD4^+$ T cell response, kinetics of FV-specific $CD4^+$ T cells were followed for 35 days after FV-infection and transfer of 10^6 or 10^7 EF4.1 TCRβ-transgenic $CD4^+$ T cells. At the peak of FV-specific $CD4^+$ T cell expansion, day 7 after infection, the number of FV-specific $CD4^+$ T cell numbers had increased 100-fold compared to the number of cells engrafted in the spleen at day 1 post infection. FV-specific donor $CD4^+$ T cell numbers subsequently declined between days 7 and 14 post infection and had reached a plateau by day 35 after infection. This precursor-determined elevated level of expansion was maintained throughout the course of infection, and the rate of FV-specific $CD4^+$ T cell contraction between day 7 and day 35 post infection was comparable whether mice had received 10^6 or 10^7 EF4.1 TCRβ-transgenic $CD4^+$ T cells (**Figure 4.1C**).

These data demonstrated that precursor number did not have a limiting effect on the ongoing FV-specific $CD4^+$ T cell response, and FV-specific $CD4^+$ T cells were not lost at a higher rate when precursor number was increased.

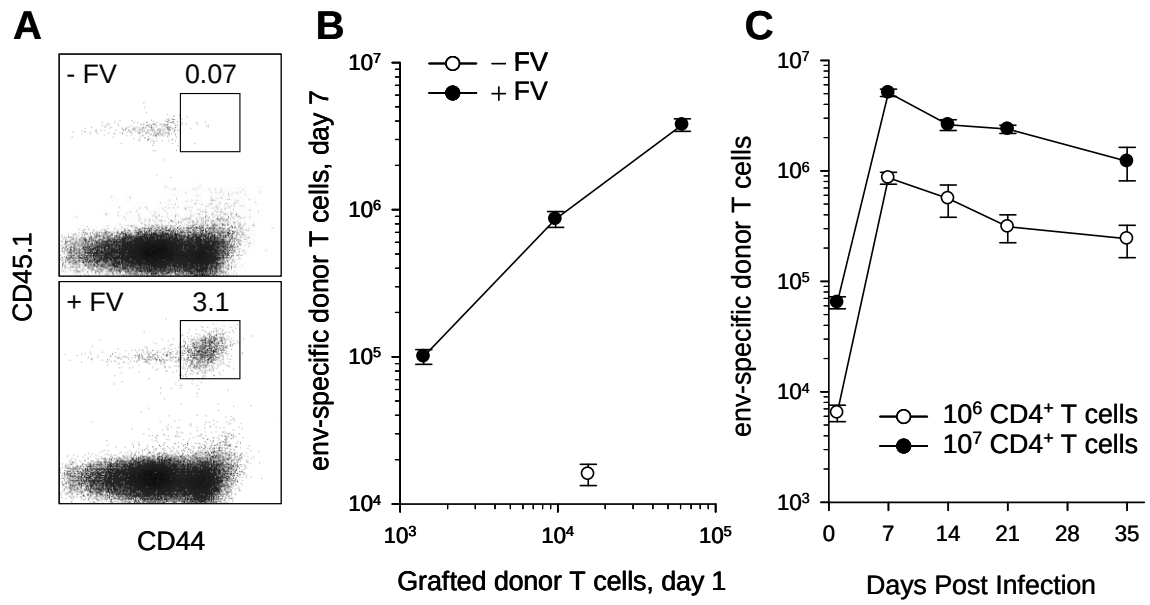

Figure 4.1 Priming of adoptively transferred TCRβ-transgenic FV-specific CD4$^+$ T cells during FV infection

(A) 1 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 (CD45.2$^+$) recipients, which remained uninfected (-FV) or were infected with FV 1 day later (+FV). The percentage of activated donor (CD44$^+$ CD45.1$^+$) CD4$^+$ T cells in gated total splenic CD4$^+$ T cells 7 days post infection is shown.

(B) Correlation between numbers of grafted FV-specific CD4$^+$ T cells in the spleen 1 day after transfer and numbers of the same cells 7 days post infection with FV (+FV). Numbers of FV-specific cells recovered from uninfected mice (-FV) are also shown for comparison. Values are the mean (± the SEM) of 3-8 mice per group per CD4$^+$ T cell inoculum from 1-3 separate experiments.

(C) 10^6 or 10^7 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 recipients one day before FV infection. Numbers of FV-specific CD4$^+$ T cells recovered from mice are shown. Values are the mean (± the SEM) of 3-8 mice per CD4$^+$ T cell inoculum from 2-3 separate experiments.

4.2.2 Effect of antigen dose on kinetics of FV-specific CD4$^+$ T cells

Upon interaction with antigen presented in complex with MHC class II molecules on APCs, CD4$^+$ T cells proliferate and acquire effector function. In order to investigate the relationship between antigen dose and expansion and contraction of FV-specific CD4$^+$ T cells, three systems were used; infection of B6 mice with F-MuLV-N (**Figure 4.2A**), infection of B6 mice with FV (**Figure 4.2B**) and infection of B6.A-*Fv2*^{s} mice with FV (**Figure 4.2C**). Mice received 2 × 10^6 TCRβ-transgenic CD4$^+$ T cells one day before infection. These systems provided conditions where antigen level was low, intermediate or high, respectively.

After infection with F-MuLV-N, an N-tropic strain of F-MuLV which is attenuated in B6 mice due to the *Fv1*^{b} allele, there was a very low percentage of infected erythroid precursor cells in the spleen at the peak of infection. This low level of initial infection was resolved rapidly, with the fraction of infected cells below the level of detection by flow cytometry by day 14 after infection. At day 7 post infection FV-specific CD4$^+$ T cells had expanded ~60-fold compared to the number of FV-specific CD4$^+$ T cells engrafted at day 1 post infection. FV-specific CD4$^+$ T cell numbers subsequently contracted, with an elevated rate of contraction between days 21 and 35 post infection (**Figure 4.2A**). Thus, the low level of antigen present after infection with F-MuLV-N was not adequate to induce a prolonged FV-specific CD4$^+$ T cell response to FV in B6 mice.

In contrast to infection with F-MuLV-N, B6 mice are permissive to infection by B-tropic F-MuLV. However, due to resistance at the *Fv2* locus (*Fv2*^{r}), B6 mice are not

susceptible to FV-induced splenomegaly and erythroleukaemia. FV infection resulted in around 1% infected cells at the peak (day 7 post infection) and infection was subsequently resolved, decreasing below the level of detection by flow cytometry by day 35 post infection as shown above. The number of FV-specific $CD4^+$ T cells increased ~70-fold at the peak of infection and, although they subsequently began to contract as observed after F-MuLV-N infection, it was at a reduced rate with cell numbers reaching a plateau between days 21 and 35 post infection (**Figure 4.2B**). From these data it was clear that at a higher level of antigen, a proportionally increased and longer-lived FV-specific $CD4^+$ T cell response could be induced.

Finally, B6.A-*Fv2*^{s} mice are permissive to infection by B-tropic F-MuLV but are also susceptible to FV-induced splenomegaly and erythroleukaemia due to the *Fv2* allele (*Fv2*^{s}). Infection is more severe than that observed in B6 mice, with 10-fold more infected cells seen at the peak of infection. In addition to this elevated level of antigen, a >200-fold expansion of FV-specific $CD4^+$ T cells was seen compared to the number of TCRβ-transgenic $CD4^+$ T cells recovered at day 1 post infection. FV-specific $CD4^+$ T cells subsequently contracted and, with kinetics similar to those seen in B6 mice, reached a plateau by day 35 post infection (**Figure 4.2C**). These results showed that increased antigen was able to induce an increased peak expansion of FV-specific $CD4^+$ T cells, but was not able to maintain these cells at levels higher than that seen in FV infection of B6 mice, despite an increased initial level of antigen. A direct correlation between the mean percentage of infected cells in the spleen at the peak of infection, and the mean number of FV-specific donor $CD4^+$ T cells recovered at the peak of expansion was also apparent in each of the three systems described above (**Figure 4.3**).

In conclusion, expansion of FV-specific donor $CD4^+$ T cells appeared to be directly proportional to the amount of antigen in the system. Hence, the magnitude of the FV-specific $CD4^+$ T cell response was antigen dose-dependent. While low level antigen was unable to maintain an FV-specific $CD4^+$ T cell response, higher levels of antigen could maintain higher numbers of these cells throughout FV infection. However, FV-specific $CD4^+$ T cell loss was not alleviated by the increased amount of antigen present during the initial FV infection of B6.A-*Fv2*^{s} mice. Together, these data demonstrated that the magnitude of the anti-retroviral FV-specific $CD4^+$ T cell response was determined both by precursor $CD4^+$ T cell number and the amount of initial antigen stimulation that donor $CD4^+$ T cells had experienced.

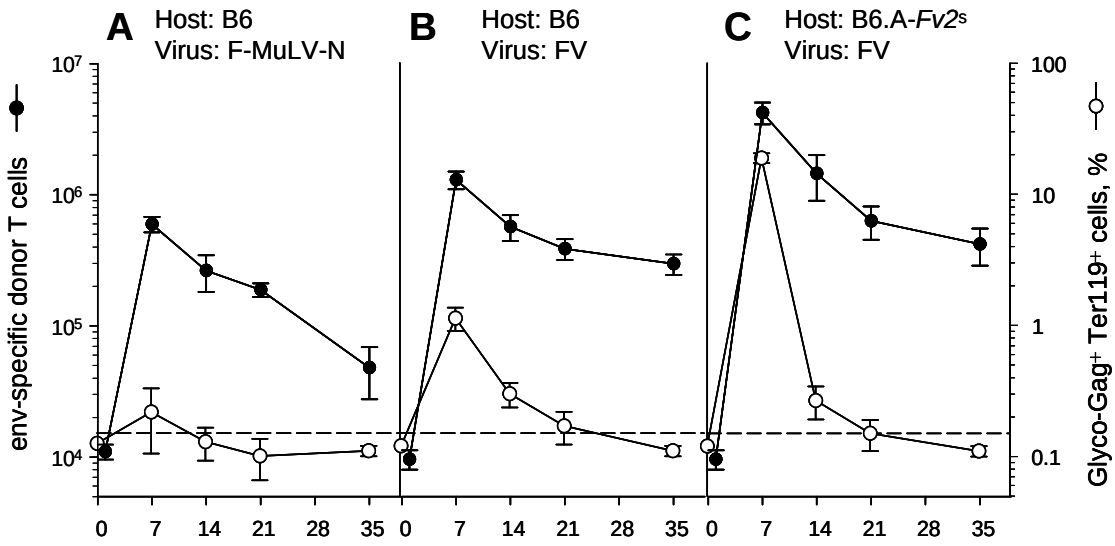

Figure 4.2 Relationship between antigen dose and FV-specific CD4$^+$ T cell expansion and contraction

(A) 2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 (CD45.2$^+$) recipients, which were infected with F-MuLV-N 1 day later. Numbers of FV-specific CD4$^+$ T cells recovered from mice and the percentage of glyco-Gag$^+$Ter119$^+$ cells in the spleen are shown. Values are the mean (± the SEM) of 6-10 mice from 2-3 separate experiments.

(B) 2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 (CD45.2$^+$) recipients, which were infected with FV 1 day later. Numbers of FV-specific CD4$^+$ T cells recovered from mice and the percentage of glyco-Gag$^+$Ter119$^+$ cells in the spleen are shown. Values are the mean (± the SEM) of 10-16 mice from 3-5 separate experiments.

(C) 2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6.A-*Fv2*^{s} (CD45.2$^+$) recipients, which were infected with FV 1 day later. Numbers of FV-specific CD4$^+$ T cells recovered from mice and the percentage of glyco-Gag$^+$Ter119$^+$ cells in the spleen are shown. Values are the mean (± the SEM) of 5-9 mice from 2-5 separate experiments.

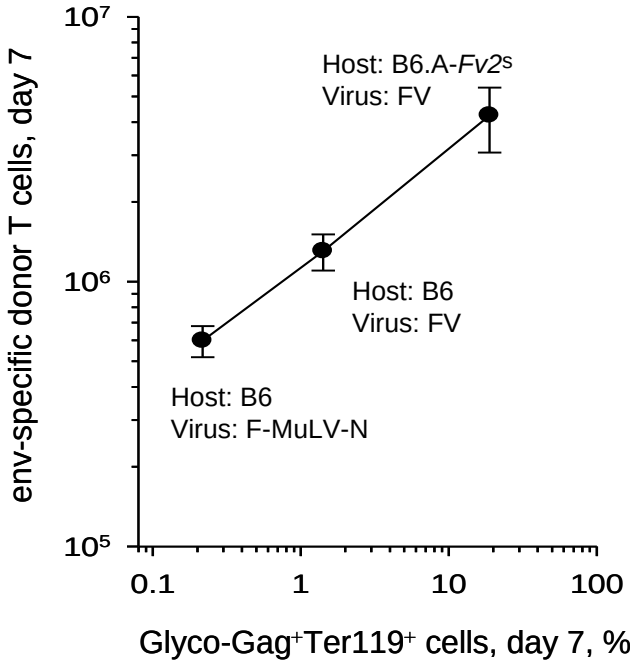

Figure 4.3 Relationship between peak expansion of FV-specific CD4$^+$ T cells and peak level of infection

Correlation between the peak percentage of FV-infected (glyco-Gag$^+$) Ter119$^+$ cells 7 days after infection of the indicated host and virus and the numbers of FV-specific CD4$^+$ T cells recovered at the same time point. Values are the mean (± the SEM) of 5-10 mice from 2-4 separate experiments per virus/host combination.

4.3 Detecting FV During the Chronic Phase of Infection

Flow cytometric detection of infected erythroid precursor cells (glyco$^-$Gag$^+$Ter119$^+$ cells) in the spleen is sufficient to differentiate between levels of infection during the acute phase in the three systems described above. However, by day 35 after infection the percentage of infected erythroid precursor cells is below detection by flow cytometry, even in B6.A-*Fv2*^{s} mice despite more severe infection and high antigen level. FV is known to establish a persistent, chronic infection (Chesebro et al., 1979). With this in mind, a Q-PCR assay that could measure the number of proviral copies of F-MuLV *env* gene per 100,000 nucleated spleen cells was developed in order to provide a more sensitive technique with which to detect FV infection during the chronic phase.

Using this assay, the number of copies of F-MuLV *env* per 100,000 cells in the spleen of acutely infected B6 mice (day 7 post infection) was compared to that during chronic infection (day 35 post infection) in the three systems described above. B6 mice infected with F-MuLV-N, which had also received EF4.1 TCRβ-transgenic CD4$^+$ T cells, had undetectable levels of F-MuLV *env*, even with the more sensitive assay. This is in agreement with the reported inability of F-MuLV-N to persist in B6 mice (Dittmer et al., 1998). In contrast, in B6 and B6.A-*Fv2*^{s} mice which had received EF4.1 TCRβ-transgenic CD4$^+$ T cells one day before FV infection, F-MuLV env was still detectable by day 35 after infection (**Figure 4.4**). However, the difference between the number of F-MuLV *env* copies per 100,000 nucleated spleen cells during chronic infection in B6 and B6.A-*Fv2*^{s} mice was not significant.

These data showed that B6 and B6.A-*Fv2*^{s} mice, despite a significant reduction in infected cells during the acute phase, still had detectable levels of F-MuLV env during chronic infection, suggesting that although additional FV-specific CD4^{+} T cells were able to reduce the peak level of infection, they were not sufficient to clear FV infection completely and a persistent chronic infection was still established.

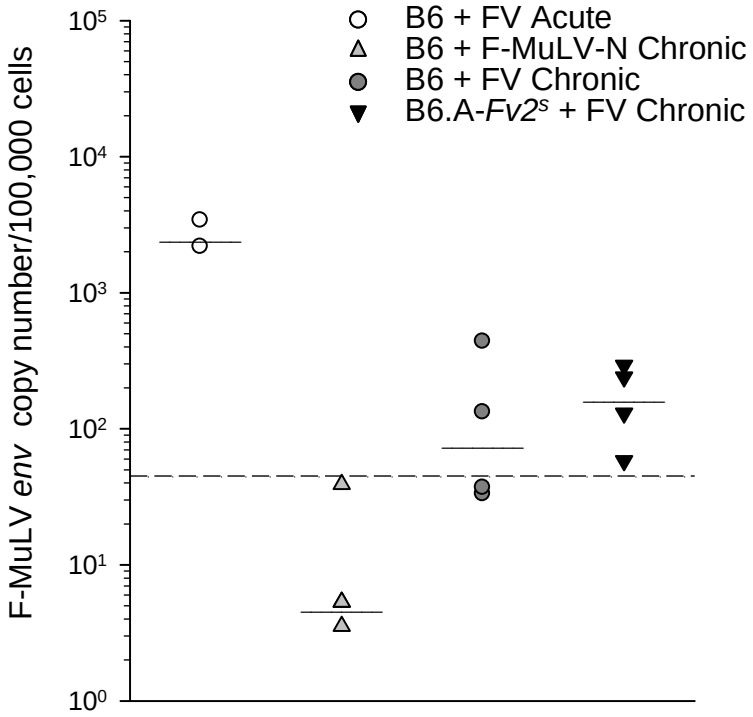

Figure 4.4 Detection of F-MuLV *env* in the spleen by Q-PCR

2×10^6 TCRβ-transgenic $CD4^+$ T cells were transferred into the indicated host and were infected with the indicated virus 1 day later. DNA was extracted from spleens of mice 35 days after FV infection and Q-PCR to detect F-MuLV *env* was carried out. For acute infection, DNA was extracted from the spleen of B6 mice 7 days after FV infection. The F-MuLV *env* copy number per 100,000 nucleated spleen cells is plotted. Each symbol represents an individual mouse. Solid lines represent the means of each group. The dashed line indicates the numbers of copies of F-MuLV *env* per 100,000 spleen cells in uninfected mice as detected by Q-PCR.

4.4 Discussion

4.4.1 Expansion of virus-specific $CD4^+$ T cells in the FV mouse model

In previous studies investigating $CD4^+$ T cell responses to viral infection, increasing the number of transgenic virus-specific $CD4^+$ T cell precursors resulted in a compromised survival of these cells, with cells being lost more rapidly when higher numbers were transferred into mice than when lower numbers were transferred. An example of this is the elevated rate of T cell loss observed when 10^5 LCMV-specific $CD4^+$ T cells were transferred compared to when only 10^3 cells were transferred. This increased loss was attributed to competition for IFN-γ signals by $CD4^+$ T cells, as this difference in loss was not observed when the transgenic $CD4^+$ T cells did not express the IFN-γR (Whitmire et al., 2008). This phenomenon was also seen in VSV infection, although in this model T cell loss was attributed to competition by $CD4^+$ T cells for antigen (Blair and Lefrancois, 2007).

The effect of increased precursor number on expansion and contraction of virus-specific $CD4^+$ T cells was assessed here using EF4.1 TCRβ-transgenic $CD4^+$ T cells in the FV model. In contrast to the above described studies, increasing the number of transgenic $CD4^+$ T cells adoptively transferred did not result in accelerated loss of FV-specific $CD4^+$ T cells. Instead, the rate of cell loss was comparable regardless of the number of cells initially transferred. In addition, the level of peak expansion continued to increase even when 10^7 EF4.1 TCRβ-transgenic $CD4^+$ T cells were transferred. Thus, it can be concluded that intial expansion of FV-specific $CD4^+$ T cells in the model used in this thesis is not limited by either antigen or IFN-γ, and that

these $CD4^+$ T cells are still receiving adequate stimulation for survival and memory cell generation even when the precursor number is higher.

The difference in the results shown here using the FV model compared to LCMV and VSV studies may also reflect a difference in the virus-specific $CD4^+$ T cell response in retroviral infection compared to non-retrovirus viral infection. However, it is necessary to draw attention to the monoclonal character of the transgenic $CD4^+$ T cells used in the two previous studies described above. The EF4.1 TCRβ-transgenic mouse used in the studies here have a polyclonal $CD4^+$ T cell population, and hence adoptive transfer experiments to study the virus-specific $CD4^+$ T cell response carried out here are of a more physiological nature than models where transgenic $CD4^+$ T cells were monoclonal. This indicates that the results presented here are likely to be more reliable than those from previous studies, and the use of the FV retroviral complex suggests that these results can be more reliably extrapolated to $CD4^+$ T cell responses in human retroviral infections.

4.4.2 Antigen-dependent virus-specific $CD4^+$ T cell expansion

Further to the results presented here regarding the non-limiting effect of antigen on precursor number compared to previous studies, the role of antigen dose on the expansion and contraction of FV-specific $CD4^+$ T cells was examined by studying the $CD4^+$ T cell response in three conditions where antigen level was low, intermediate or high. While, low levels of antigen were not adequate to induce and maintain a strong FV-specific $CD4^+$ T cell response, high levels of antigen induced a strong FV-specific $CD4^+$ T cell response which did not decline as rapidly as when the antigen level was lower.

These data demonstrated that the expansion of FV-specific $CD4^+$ T cells is antigen dose-dependent, and thus that antigen is a limiting factor for the expansion of FV-specific $CD4^+$ T cells. It further showed that during conditions where the level of antigen is high, for example in B6 mice, cells are not rapidly deleted due to overactivation by antigen during a persistent viral infection. This is in contrast to studies which observed $CD8^+$ T cell loss during chronic persistent infection by LCMV (Fuller et al., 2004; Moskophidis et al., 1993).

4.4.3 Infection with an attenuated F-MuLV is unable to induce a strong or long-lived FV-specific CD4$^+$ T cell response

Previous studies concluded that CD4$^+$ T cells had no role in protecting against FV infection after absence of protection in mice which received CD4$^+$ T cells from immune mice which had been immunised with attenuated F-MuLV (Dittmer and Hasenkrug, 2000). Here, availability of the EF4.1 TCRβ-transgenic mouse has allowed detection and quantification of the FV-specific CD4$^+$ T cell response after both infection and immunisation. Employing this model, it is shown here that although FV-specific CD4$^+$ T cells initially expanded in response to attenuated F-MuLV vaccination, there was a more rapid contraction of virus-specific CD4$^+$ T cells than that observed after WT FV infection. This shows that the inability to confer protection by transfer of CD4$^+$ T cells from immune mice in a previous study likely occurred because vaccination with attenuated F-MuLV only induced a weak FV-specific CD4$^+$ T cell response, and this FV-specific CD4$^+$ T cell response was long-lasting FV-specific memory CD4$^+$ T cells. Therefore, the results and conclusions regarding the lack of requirement for CD4$^+$ T cells in FV infection may be dismissed.

In contrast to the findings here, studies which used SIV as a model for HIV showed that infection with attenuated SIV was able to induce a functional SIV-specific effector-memory CD4$^+$ T cell response (Gauduin et al., 2006). The different results seen in this previous study compared to the experiment described here may lie in the different degree of attenuation if the viruses used. As the attenuated F-MuLV did not induce an FV-specific CD4$^+$ T cell response as strong as the wild-type virus, it is possible that it is too attenuated, and that a virus with a lower degree or different mechanism of attenuation would be able to induce an improved CD4$^+$ T cell response.

4.4.4 An alternative assay for detection of low level FV infection

Flow cytometry to detect infected erythroid precursor cells in the spleen of B6 mice is a method of adequate sensitivity to measure levels of FV in acute infection and to assess the effect of transfer of EF4.1 TCRβ-transgenic $CD4^+$ T cells on protection during the acute phase. However by day 14 after infection, the percentage of infected erythroid precursors in the spleen is below the level of detection by flow cytometry. A Q-PCR assay with which to measure the number of proviral copies of F-MuLV *env* in all spleen cells provides a more sensitive method by which to quantify FV infection when the level of infection is low, for example during the chronic phase of infection when acute infection has been resolved but the virus has not been completely cleared. This further allowed assessment of the effect of elevated numbers of FV-specific $CD4^+$ T cells during FV infection, as demonstrated in chapter 6.

Chapter Five

5 Mechanisms of High Avidity FV-specific $CD4^+$ T cell Loss

5.1 Introduction

It has been demonstrated *in vitro* that the FV-specific $CD4^+$ T cell population in TCRβ-transgenic B6.EF4.1 mice contains virus-specific $CD4^+$ T cells of different avidities, with high avidity $CD4^+$ T cells defined by their use of the Vα2 TCR chain ($V\alpha2^+$) (Antunes et al., 2008). During *in vivo* FV infection in immunocompetent mice, there is a preferential expansion of these high avidity $V\alpha2^+$ FV-specific $CD4^+$ T cells to constitute >60% of responding $CD4^+$ T cells at the peak of infection. However, this expansion is followed by a rapid loss of high avidity cells, faster than that of low avidity cells. By day 35 after infection, clonal composition of the virus-specific $CD4^+$ T cell pool is the comparable to that seen in virus-naive EF4.1 TCRβ-transgenic $CD4^+$ T cells (Ploquin et al., manuscript submitted.).

To survive as memory cells, $CD4^+$ T cells require continued stimulation from antigen and cytokines, and insufficient stimulation and signalling can compromise their survival within the memory cell pool (Lanzavecchia and Sallusto, 2005). Furthermore, a number of factors can actively contribute to and promote loss of virus-specific T cells, including chronic antigen stimulation leading to deletion, or direct infection of virus-specific cells. Here, the potential mechanisms underlying the loss of high avidity virus-specific $CD4^+$ T cell in the FV mouse model were elucidated, and ways in which to maintain an FV-specific response with an elevated level of high avidity virus-specific $CD4^+$ T cells were investigated.

5.2 Infection of FV-specific CD4$^+$ T Cells by FV

HIV, which infects and kills CD4$^+$ T cells, has been shown to preferentially infect HIV-specific CD4$^+$ T cells, contributing to loss of the HIV-specific immune response (Douek et al., 2002; Mattapallil et al., 2005). FV has been shown to infect cells via the cationic amino-acid transporter (CAT-1), which is expressed on most or all murine cells (Wang et al., 1991). T cells are therefore potential targets for FV infection, and similar to observed HIV tropism, FV-specific T cells may be infected preferentially. Here the effect of direct infection of FV-specific CD4$^+$ T cells and its potential contribution to loss of high avidity Vα2$^+$ FV-specific CD4$^+$ T cells is investigated.

To determine whether direct infection of FV-specific CD4$^+$ T cells by FV was causing selective loss of the high avidity Vα2$^+$ FV-specific CD4$^+$ T cell population, the role of the *Fv1* gene in conferring permissiveness to infection was exploited by transferring TCRβ-transgenic CD4$^+$ T cells with different susceptibility to FV infection. Mice received *Fv1*^{b} TCRβ-transgenic CD4$^+$ T cells, which are permissive to infection by B-tropic FV, or *Fv1*^{n} TCRβ-transgenic CD4$^+$ T cells, which are not permissive to infection by B-tropic FV, in a 1:1 ratio. (NB: TCRβ-transgenic CD4$^+$ T cells used in all other experiments are *Fv1*^{b}). In order to identify the separate populations, *Fv1*^{b} cells were CD45.1$^+$, while *Fv1*^{n} cells were CD45.1/2$^+$. Cells were transferred into CD45.2$^+$ B6 hosts. When analysed by flow cytometry, endogenous host CD4$^+$ T cells (CD45.2$^+$), donor *Fv1*^{b} FV-specific CD4$^+$ T cells (CD45.1$^+$) and donor *Fv1*^{n} FV-specific CD4$^+$ T cells (CD45.1/CD45.2$^+$) could be identified, and the clonal composition of each population could be analysed (**Figure 5.1A**). Donor CD4$^+$ T cell kinetics were followed to determine whether there was a preferential loss of FV-

infection permissive *Fv1*^{b} FV-specific CD4^{+} T cells, particularly, the high avidity population.

Expansion and contraction of both *Fv1*^{b} and *Fv1*^{n} FV-specific donor CD4^{+} T cells after FV infection were comparable, and there was no preferential loss of cells which had susceptibility to FV infection (**Figure 5.1B**). Furthermore, when the kinetics of high avidity Vα2^{+} cells were examined in *Fv1*^{n} FV-specific donor CD4^{+} T cells and *Fv1*^{b} FV-specific donor CD4^{+} T cells, no difference was observed in the rate of expansion or contraction of Vα2^{+} cells (**Figure 5.1C**). Therefore, FV-specific CD4^{+} T cells that were not able to be infected by FV do not have a survival advantage over those which potentially can be FV-infected. This demonstrated that loss of FV-specific CD4^{+} T cells, and in particular selective loss of high avidity FV-specific CD4^{+} T cells, was not due to direct cytopathic infection of virus-specific CD4^{+} T cells in the FV model.

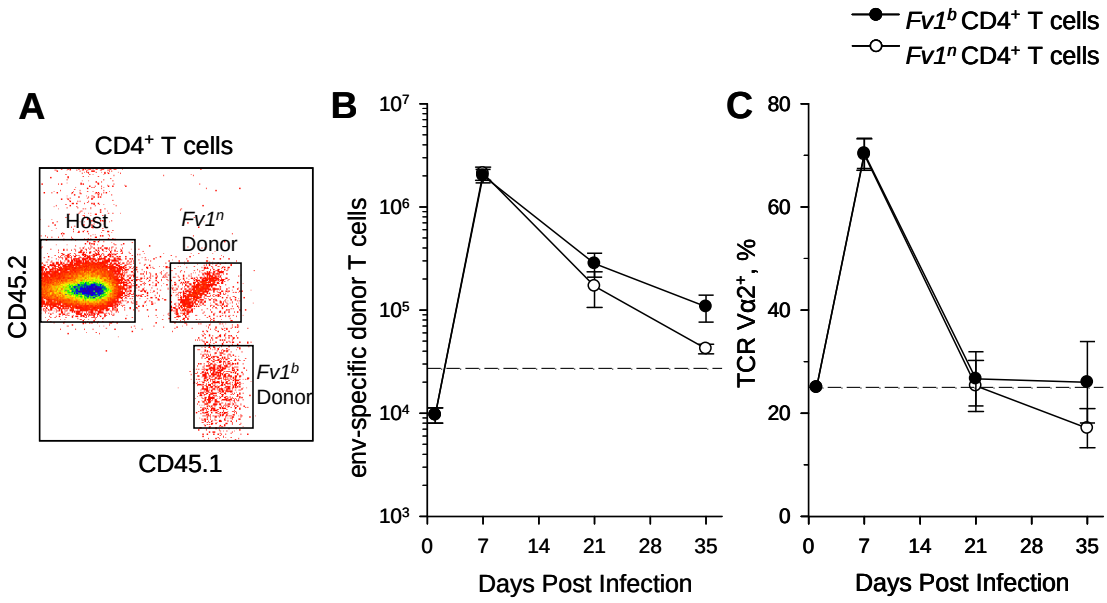

Figure 5.1 Kinetics and clonal composition of FV-specific TCRβ-transgenic CD4$^+$ T cells that are permissive or not to infection by FV

2 × 10^6 CD45.1$^+$ *Fv1^b* TCRβ-transgenic CD4$^+$ T cells and 2 × 10^6 CD45.1/2$^+$ *Fv1^n* TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice (CD45.2$^+$) 1 day before FV infection.

(A) Host CD4$^+$ T cells, CD45.1$^+$ *Fv1^b* donor CD4$^+$ T cells, and CD45.1/2$^+$ *Fv1^n* donor CD4$^+$ T cells are gated.

(B) Numbers of CD45.1$^+$ *Fv1^b* or CD45.1/2$^+$ *Fv1^n* FV-specific CD4$^+$ T cells recovered after infection are shown. The dashed line indicates maximum donor cell expansion in the absence of infection.

(C) Percentages of CD45.1$^+$ *Fv1^b* or CD45.1/2$^+$ *Fv1^n* FV-specific CD4$^+$ T cells expressing TCR Vα2 are shown. The dashed line indicates the percentage of Vα2$^+$ cells seen in the donor cell population.

Values are the mean (± the SEM) of 5 mice per group per time point from 2 separate experiments.

5.3 Activation of High and Low Avidity FV-specific $CD4^+$ T Cells

FV-specific $CD4^+$ T cells were defined by expression of CD44, a permanent marker of cell activation. CD44 continues to be expressed on the cell surface even when a cell is no longer activated and is a marker of memory $CD4^+$ T cells. In contrast, CD43 is transiently expressed when a cell is activated, and downregulated when cells cease to be activated. Expression of CD43 on $CD44^+$ donor $CD4^+$ T cells in response to FV infection was assessed.

At the peak of infection and FV-specific $CD4^+$ T cell expansion, day 7 post infection, CD43 expression correlated with CD44 expression, and the percentage of $V\alpha2^+$ or $V\alpha2^-$ FV-specific $CD4^+$ T cells expressing CD43 was comparable, verifying the use of CD44 as a marker to identify activated virus-specific $CD4^+$ T cells at the peak of expansion. (**Figure 5.2**). However, fewer $CD44^{hi}$ cells were expressing CD43 at day 14 after infection, indicating that cells were mainly activated at the peak of FV infection and suggesting that CD43 would provide a more reliable marker to assess activation of FV-specific $CD4^+$ T cells during the later stage of FV infection. Notably, at day 14 after infection, the $V\alpha2^+$ FV-specific $CD4^+$ T cell population contained significantly more CD43 expressing cells than the $V\alpha2^-$ population ($p=0.008$). This indicates that the $V\alpha2^+$ FV-specific $CD4^+$ T cell population did not express more CD43 than the $V\alpha2^-$ population at the peak of infection, but retained CD43 expression after this and hence was fully activated for longer.

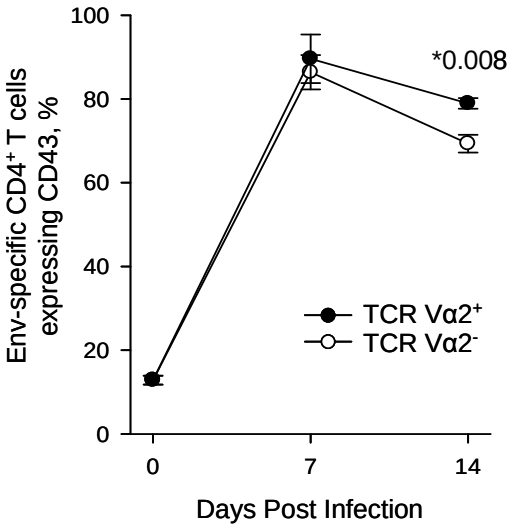

Figure 5.2 Expression of CD43 on high and low avidity FV-specific CD4$^+$ T cells during FV infection

2×10^6 CD45.1$^+$ EF4.1 TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice (CD45.2$^+$) 1 day before FV infection. Percentage of Vα2$^-$ FV-specific donor CD4$^+$ T cells and Vα2$^+$ FV-specific donor CD4$^+$ T cells expressing CD43 during FV infection. Values are (± the SEM) of 2-5 mice per group per time point from 2 separate experiments. The number within the graph denotes the *p* value as compared using a two-tailed student's t-test.

5.4 Cytokine Profiles of High and Low Avidity FV-specific $CD4^+$ T Cells

Lanzavecchia and Sallusto described a model of progressive differentiation whereby cells express a different range of cytokines relative to the amount of antigen stimulation received (Lanzavecchia and Sallusto, 2005). It is shown here in section **3.4.1** that FV-specific $CD4^+$ T cells had a predominantly Th1 phenotype, producing IFN-γ and IL-2, but negligible IL-17. With this in mind, the percentage of IL-2 single producers, IFN-γ single producers, and IL-2/IFN-γ double producers in the $V\alpha2^-$ and $V\alpha2^+$ FV-specific $CD4^+$ T cell populations were compared in order to assess whether the high avidity population was more differentiated than the low avidity population.

The percentage of $V\alpha2^+$ FV-specific $CD4^+$ T cells producing IL-2 alone was equivalent to that seen in the $V\alpha2^-$ population (**Figure 5.3A**). There was a trend towards an increased proportion of IFN-γ single producers in the $V\alpha2^+$ population but this difference was not statistically significant (**Figure 5.3C**). Notably, there were significantly more FV-specific $CD4^+$ T cells producing both IL-2 and IFN-γ in the $V\alpha2^+$ population compared to the $V\alpha2^-$ population ($p=0.04$) (**Figure 5.3B**). These data showed that a higher proportion of high avidity FV-specific $CD4^+$ T cells were producing cytokines compared to the low avidity FV-specific $CD4^+$ T cell population. However, although the high avidity population contained more double cytokine-producers than the low avidity, the overall phenotype of high avidity cells did not indicate a high level of differentiation. Therefore, high avidity FV-specific $CD4^+$ T cells were not likely to be lost preferentially over low avidity cells due to terminal differentiation.

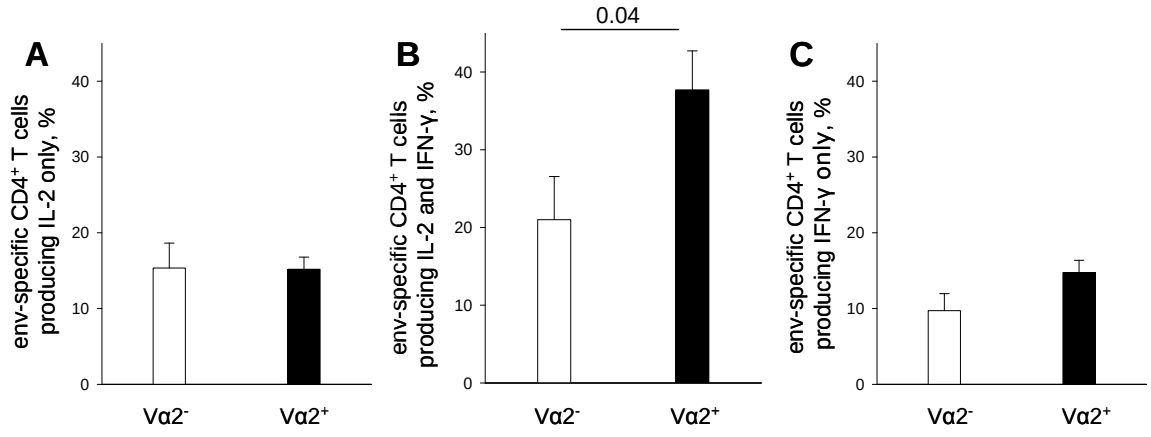

Figure 5.3 Cytokine production by high and low avidity FV-specific CD4$^+$ T cells after FV infection

2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice (CD45.2$^+$) 1 day before FV infection.

(A) Percentage of IL-2-producing Vα2$^-$ and Vα2$^+$ FV-specific donor CD4$^+$ T cells 7 days after FV infection.

(B) Percentage IFN-γ and IL-2 double-producing FV-specific donor CD4$^+$ T cells 7 days after FV infection.

(C) Percentage of IFN-γ-producing Vα2$^-$ and Vα2$^+$ FV-specific donor CD4$^+$ T cells 7 days after FV infection.

Values are the mean (+ the SEM) of 6 mice per group from 2 separate experiments.

The number within the graph denotes the *p* value as compared using a two-tailed student's t-test.

5.5 Homeostatic Cytokine Receptor Expression on high and low avidity FV-specific CD4$^+$ T Cells

In order to survive and thrive in the memory pool, CD4$^+$ T cells require signals from homeostatic cytokines including IL-7, IL-15 and IL-2 (Lanzavecchia and Sallusto, 2005). To receive survival signals from these cytokines it is necessary to express the appropriate cell surface receptor and a deficiency in homeostatic cytokine receptors may result in a compromised ability of the memory T cell to survive. Expression of the IL-2 receptor α chain (IL-2Rα), the IL-2 receptor β chain/IL-15 receptor α chain (IL-2Rβ/IL-15Rα) and the IL-7 receptor α chain (IL-7Rα) were studied and the proportion of high and low avidity FV-specific CD4$^+$ T cells expressing these receptors was compared.

The proportion of cells expressing IL-2Rα followed expected kinetics during the course of infection, with ~3% of cells expressing it at day 1 post infection, then an increase to ~10% as cells entered effector stage. While the proportion of Vα2$^-$ FV-specific CD4$^+$ T cells expressing IL-2α had declined by day 14, the proportion of Vα2$^+$ FV-specific CD4$^+$ T cells continued to rise, and at day 14 after infection the population contained significantly more IL-2Rα expressing cells than the Vα2$^-$ population ($p=0.008$) (**Figure 5.4A**). This suggested that potentially high avidity Vα2$^+$ cells had an enhanced ability to receive signals from IL-2, and so their loss could not be attributed to an inability to receive survival signals from IL-2. However, this difference was still minimal and so this observation may not be of biological significance.

The proportion of cells expressing IL-2Rβ/IL-15Rα also followed expected kinetics, where ~5% of cells express it initially, expression subsequently increasing in the late effector cell population, and then remaining stable as cells entered the memory $CD4^+$ T cell pool. However, there was no difference in the percentage of cells (~20% in each case) expressing IL-2Rβ/IL-15Rα when $Vα2^-$ FV-specific $CD4^+$ T cells were compared with $Vα2^+$ FV-specific $CD4^+$ T cells (**Figure 5.4B**).

Finally, the proportion of cells expressing IL-7Rα again followed expected kinetics, with ~38% of cells expressing the receptor initially. This had declined by day 7 post infection where only 12% of cells expressed IL-7Rα. At day 14 after infection, when cells had entered the memory pool, approximately 25-30% of cells were expressing IL-7Rα, and although there was a trend towards fewer $Vα2^+$ FV-specific $CD4^+$ T cells expressing the IL-7Rα compared to $Vα2^-$ cells, this difference was not significant (**Figure 5.4C**).

These data demonstrated that expression of receptors for IL-2, IL-7 and IL-15 was standard in both high and low avidity virus-specific $CD4^+$ T cell populations, and so both can effectively receive survival signals from these cytokines. Also, compared to low avidity $Vα2^-$ FV-specific $CD4^+$ T cells, high avidity $Vα2^+$ FV-specific $CD4^+$ T cells did not have a markedly lower expression of any of the homeostatic cytokine receptors, and were not lost due to a compromised ability to receive survival signals. In contrast, high avidity FV-specific $CD4^+$ T cells may actually be able to receive increased signals from IL-2.

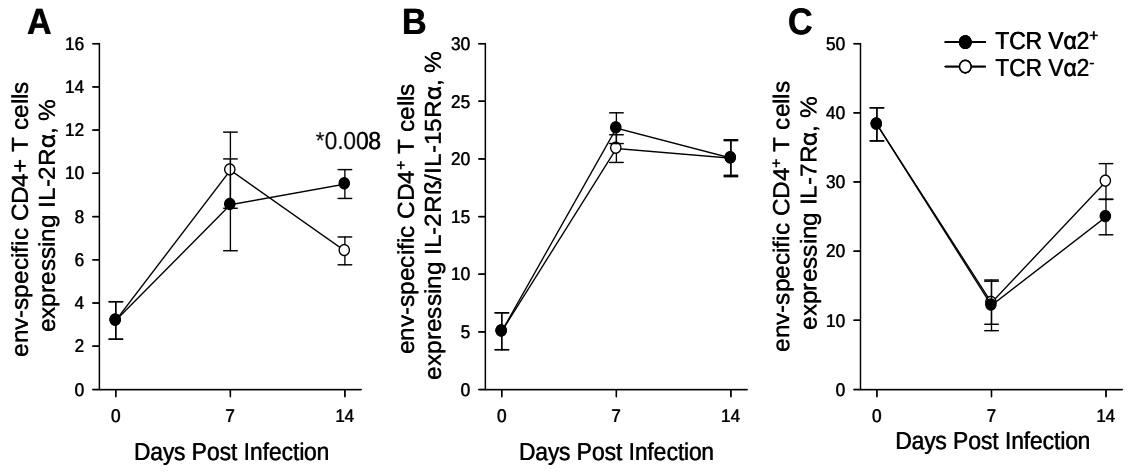

Figure 5.4 Homeostatic cytokine receptor expression on high and low avidity FV-specific CD4$^+$ T cells

2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice (CD45.2$^+$) 1 day before FV infection.

(A) Percentage of Vα2$^-$ FV-specific donor CD4$^+$ T cells or Vα2$^+$ FV-specific donor CD4$^+$ T cells expressing IL-2Rα (CD25) after FV infection.

(B) Percentage of Vα2$^-$ FV-specific donor CD4$^+$ T cells or Vα2$^+$ FV-specific donor CD4$^+$ T cells expressing IL-2Rβ/IL-15Rα (CD122) after FV infection.

(C) Percentage of Vα2$^-$ FV-specific donor CD4$^+$ T cells or Vα2$^+$ FV-specific donor CD4$^+$ T cells expressing IL-7Rα (CD127) after FV infection.

Values are (± the SEM) of 5-8 mice per group per time point from 2-3 separate experiments. The number within the graph denotes the *p* value as compared using a two-tailed student's t-test.

5.5.1 The effect antigen persistence on the loss of high avidity FV-specific $CD4^+$ T cells

The rapid decline of high avidity virus-specific $CD4^+$ T cells seen in FV infection is reminiscent of T cell loss during chronic viral infection (Moskophidis et al., 1993). Non-persisting sources of antigen which have previously been shown to induce an FV-specific T cell response, were used to examine the potential contribution of chronic and persistent antigen stimulation to the loss of high avidity $V\alpha2^+$ $CD4^+$ T cells.

5.5.2 The effect of peptide immunisation on loss of high avidity FV-specific $CD4^+$ T cells

Immunisation of mice with an $env_{124-138}$ peptide and Sigma adjuvant system provides a source of antigen which induces a memory FV-specific $CD4^+$ T cell pool adequate to reduce FV infection to a comparable level of that after transfer of naive EF4.1 TCRβ-transgenic $CD4^+$ T cells one day before FV infection, as shown above (**Figure 3.1**).

Mice received TCRβ-transgenic $CD4^+$ T cells one day before immunisation. Peptide immunisation induced a strong FV-specific $CD4^+$ T cell response, comparable to that induced by a wild-type FV infection, with FV-specific $CD4^+$ T cell expansion at the peak of infection and a subsequent decline at a steady rate (**Figure 5.5A**). High avidity $V\alpha2^+$ FV-specific $CD4^+$ T cells preferentially expanded within this population but, despite the absence of persisting antigen, returned to levels seen in virus naive cells by 35 days after infection (**Figure 5.5B**). Thus it appeared that peptide immunisation was able to induce an FV-specific $CD4^+$ T cell response initially dominated by high avidity cells, but that these cells were subsequently lost with kinetics similar to those observed in a WT infection.

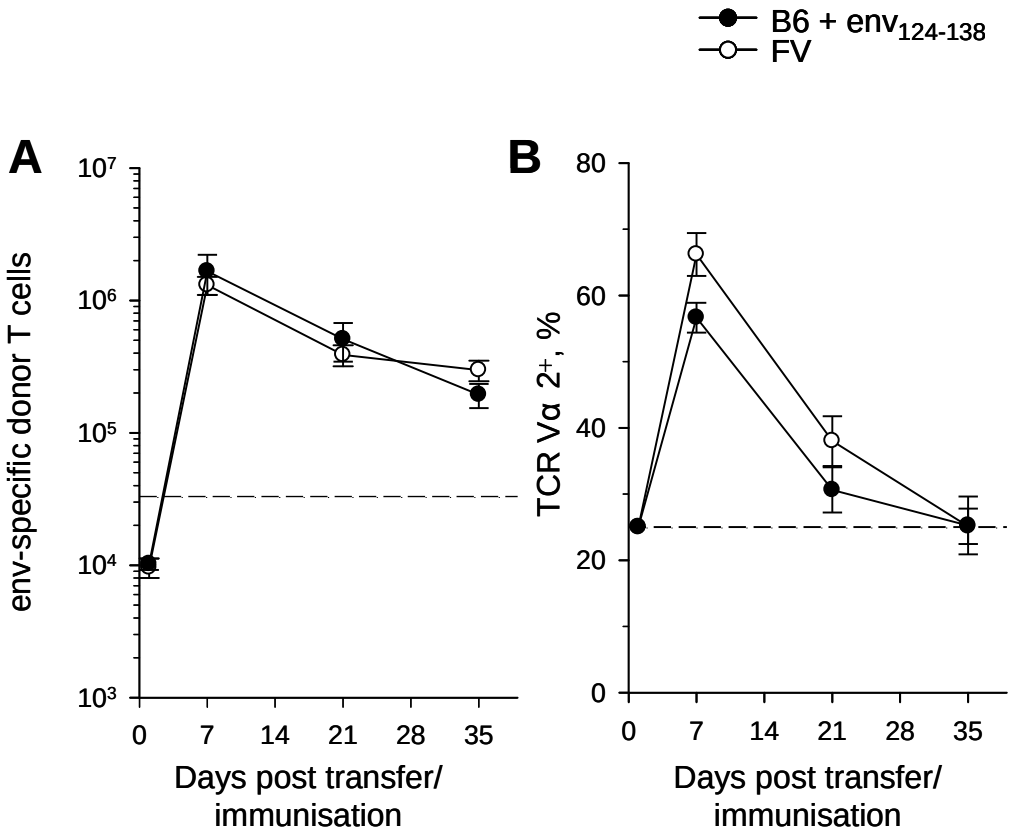

Figure 5.5 Kinetics and clonal composition of FV-specific CD4$^+$ T cells after immunisation with an F-MuLV env peptide

2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice (CD45.2$^+$) 1 day before either FV infection or immunisation with env$_{124-138}$ and adjuvant.

(A) Numbers of FV-specific donor CD4$^+$ T cells recovered after infection or immunisation are. The dashed line indicates maximum donor cell expansion in the absence of infection.

(B) Percentage of FV-specific donor CD4$^+$ T cells expressing TCR Vα2. Values are the mean (± the SEM) of 6 mice per group per time point from 3 separate experiments. The dashed line indicates the percent of Vα2$^+$ cells seen in the naive donor cell population.

5.5.3 Effect of FBL-3 cell transfer on high avidity $CD4^+$ T cell loss

To further confirm that high avidity virus-specific $CD4^+$ T cells were not being lost due to chronic antigen stimulation by persisting antigen, the FBL-3 cell line which is derived from an F-MuLV-induced leukaemia, was used to immunise mice. FBL-3 cells express FV antigens, and transfer into mice has been shown to induce a protective immune response against FV infection (Klarnet et al., 1989). FBL-3 cells are cleared within a week by gag-specific $CD8^+$ cytotoxic T cells (Chen et al., 1996).

Mice received equal numbers of TCRβ-transgenic $CD4^+$ T cells and FBL-3 cells. An FV-specific $CD4^+$ T cell response equivalent to that of the wild-type infection was induced, with expansion at day 7 post infection followed by contraction (**Figure 5.6A**). Once more, this response was dominated by high avidity $V\alpha2^+$ $CD4^+$ T cells at the peak of expansion, but these cells were subsequently lost at a higher rate than low avidity cells, returning to proportions seen in antigen naive $CD4^+$ T cells by day 35 after transfer (**Figure 5.6B**).

Together these data demonstrated that an FV-specific $CD4^+$ T cell response dominated by high avidity clones could not be maintained even in the absence of persisting antigen. Hence, chronic and persistent antigen stimulation is not responsible for the selective loss of $V\alpha2^+$ FV-specific $CD4^+$ T cells.

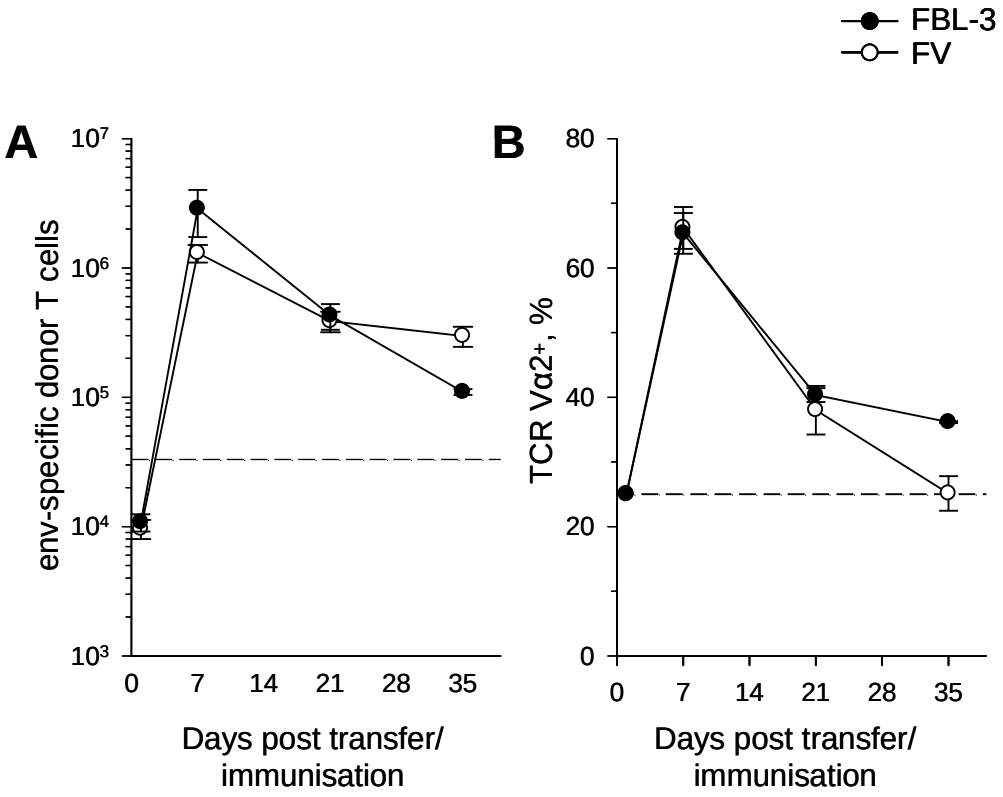

Figure 5.6 Kinetics and clonal composition of FV-specific CD4$^+$ T cells after transfer of FBL-3 cells

2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice (CD45.2$^+$) 1 day before FV infection or transfer of 2-3 × 10^6 cells from the FBL-3 F-MuLV-induced tumour cell-line.

(A) Numbers of FV-specific donor CD4$^+$ T cells recovered after infection or FBL-3 transfer are. The dashed line indicates maximum donor cell expansion in the absence of infection.

(B) Percentages of FV-specific donor CD4$^+$ T cells expressing TCR Vα2 are shown. The dashed line indicates the percentage of Vα2$^+$ cells seen in the donor cell population.

Values are the mean (± the SEM) of 2-4 mice per group per time point from 1-2 separate experiments.

5.6 Effect of Antigen Dose on High Avidity FV-specific CD4$^+$ T Cell Kinetics

As described in section **4.2.2**, there are three systems in which it is possible to study the effect of antigen dose on the CD4$^+$ T cell response; infection of B6 mice with an attenuated virus (F-MuLV-N), infection of B6 mice with FV, and infection of B6.A-*Fv2*^{s} mice with FV. These models were employed to further assess the effect of different amounts of antigen and antigen persistence on the kinetics of high avidity FV-specific CD4$^+$ T cells.

In B6 mice infected with F-MuLV-N, where antigen is at its lowest level, Vα2$^+$ cells only expanded to comprise approximately 55% of all FV-specific CD4$^+$ T cells, and thus did not expand to the levels seen in a wild-type infection, where high avidity Vα2$^+$ cells expand to comprise >60% of the total FV-specific CD4$^+$ T cell population at the peak of infection (**Figure 5.7A**). Notably, Vα2$^+$ cells returned to levels seen in naive TCRβ-transgenic CD4$^+$ T cells by day 14, sooner than after a WT infection. This showed that there was not sufficient antigen to either expand the FV-specific CD4$^+$ T cell population initially or to maintain the high avidity FV-specific CD4$^+$ T cell population beyond the peak of infection.

In the wild-type infection where B6 mice are infected with FV, Vα2$^+$ clones preferentially expanded to comprise >60% of all FV-specific CD4$^+$ T cells but were subsequently lost until they reached levels seen in the naive TCRβ-transgenic CD4$^+$ T cells by day 35 post infection (**Figure 5.7B**). In contrast, when Vα2$^+$ cell kinetics were studied after infection of B6.A-*Fv2*^{s} mice where antigen level was higher than in infection of B6 mice, the FV-specific donor CD4$^+$ T cell population contained a

higher proportion of Vα2$^+$ cells than in the wild-type infection (mean=75%). Vα2$^+$ cells were subsequently lost at a similar rate to that observed in wild-type infection. However, between day 21 and day 35 post infection they nonetheless gained a survival advantage over Vα2$^-$ cells (**Figure 5.7C**). Thus, low levels of antigen did not appear to be adequate to induce and maintain an FV-specific CD4$^+$ T cell response of high avidity. In contrast, a higher level of antigen was beneficial for initial induction of the CD4$^+$ T cell response and notably, high avidity FV-specific CD4$^+$ T cells could be maintained in the presence of persistent antigen and were not lost due to excessive antigen stimulation.

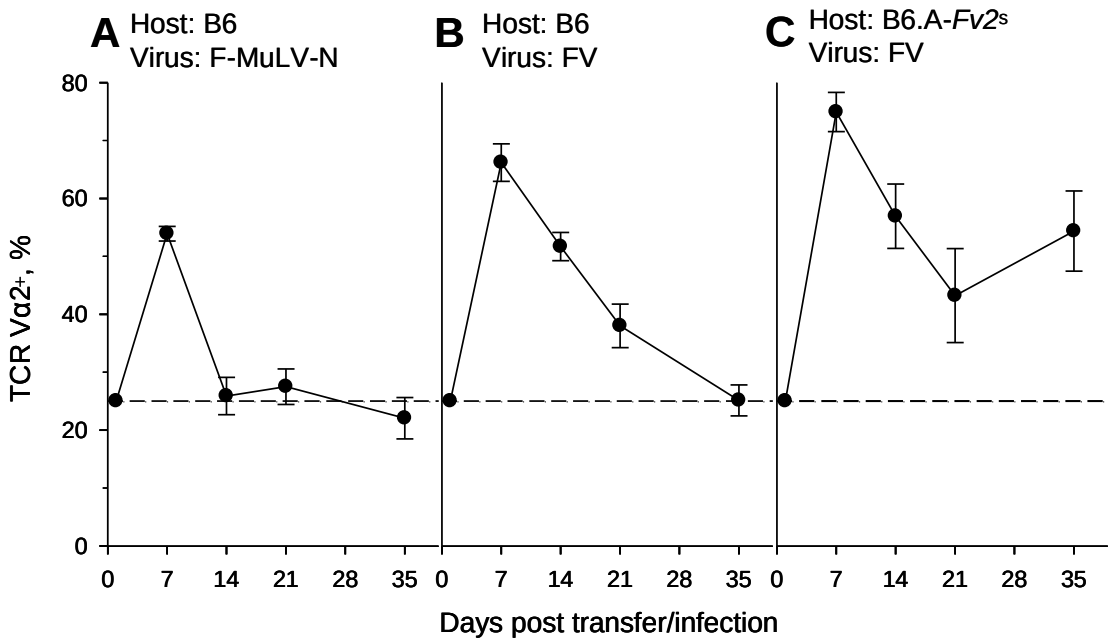

Figure 5.7 Effect of antigen dose on the loss of high avidity FV-specific TCRβ-transgenic CD4$^+$ T cells

(A) 2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 (CD45.2$^+$) recipients, which were infected with F-MuLV-N 1 day later. Percentages of FV-specific donor CD4$^+$ T cells expressing TCR Vα2 are shown. Values are the mean (± the SEM) of 6-10 mice from 2-3 separate experiments.

(B) 2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 (CD45.2$^+$) recipients, which were infected with FV 1 day later. Percentages of FV-specific donor CD4$^+$ T cells expressing TCR Vα2 are shown. Values are the mean (± the SEM) of 10-16 mice from 3-5 separate experiments.

(C) 2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6.A-*Fv2*^{s} (CD45.2$^+$) recipients, which were infected with FV 1 day later. Percentages of FV-specific donor CD4$^+$ T cells expressing TCR Vα2 are shown. Values are the mean (± the SEM) of 5-9 mice from 2-5 separate experiments.

The dashed line indicates the percentage of Vα2$^+$ cells in the naive donor cell population.

5.7 Secondary $CD4^+$ T Cell Responses in FV Infection

The data presented in sections **3.2.1** and **5.5.2** have demonstrated that immunisation with Th epitope F-MuLV env peptide was adequate to protect against FV infection, and was able to induce an FV-specific $CD4^+$ T cell response comparable in both magnitude and clonal composition to that of a WT infection in immunocompetent mice. To investigate the secondary response of virus-specific $CD4^+$ T cells to FV, mice were re-immunised or FV-infected 35 days after initial peptide immunisation.

Secondary peptide immunisation of mice induced a robust FV-specific $CD4^+$ T cell response which was long-lived, and did not undergo the rapid contraction phase seen after primary peptide immunisation (**Figure 5.8A**). In contrast, FV infection 35 days after primary peptide immunisation did not induce a response as strong as that induced either by secondary peptide immunisation or primary FV infection. These FV-specific $CD4^+$ T cells were not lost at the same rapid rate seen after primary FV infection. Instead, by day 21 the number of FV-specific $CD4^+$ T cells had reached levels seen at day 35 after initial immunisation or FV infection due to a reduced initial expansion (**Figure 5.8A**).

At the peak of expansion after secondary immunisation, the clonal composition of FV-specific $CD4^+$ T cells was similar to that seen after primary peptide immunisation, but did not contain as many $V\alpha2^+$ cells as after primary FV infection. Secondary FV infection induced an FV-specific $CD4^+$ T cell response of a similar clonal composition. However, although $V\alpha2^+$ cells in the $CD4^+$ T cell population gained a survival advantage in the response to secondary immunisation, those responding to

secondary FV infection were rapidly lost. Thus, by day 35 after infection, re-immunised mice had a higher level of $V\alpha2^+$ FV-specific $CD4^+$ T cells than mice which had been infected after primary immunisation (**Figure 5.8B**).

Together, these experiments demonstrated that a secondary $env_{124\text{-}138}$ peptide immunisation was adequate to induce a long-lasting FV-specific $CD4^+$ T cell response comprised predominantly of high avidity cells. Notably, induction of a secondary response using virus rather than F-MuLV env peptide did not induce a long-lasting FV-specific $CD4^+$ T cell response, and the remaining population contained a low proportion of high avidity clones.

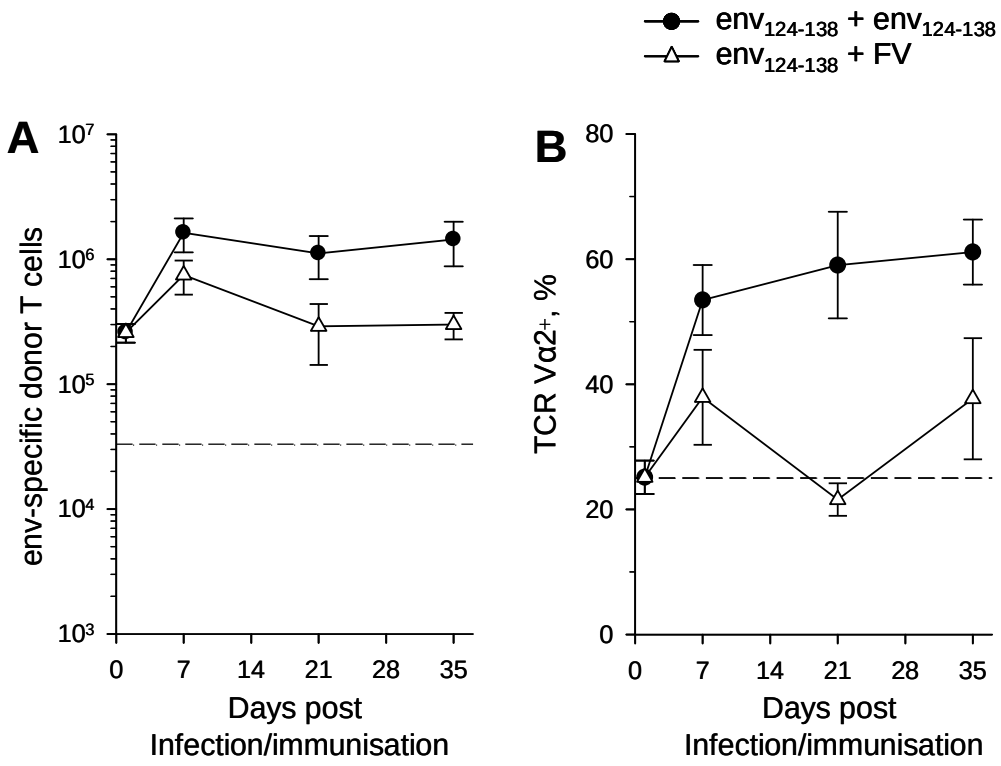

Figure 5.8 Kinetics and clonal composition of secondary FV-specific CD4$^+$ T cell responses after secondary FV infection or env peptide immunisation

2 × 10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice (CD45.2$^+$) 1 day before FV infection or immunisation with $env_{124-138}$ and adjuvant. 35 days after immunisation mice were FV infected or re-immunised.

(A) Numbers of FV-specific donor CD4$^+$ T cells recovered after secondary infection or immunisation are shown. The dashed line indicates maximum donor cell expansion in the absence of infection.

(B) Percentage of FV-specific donor CD4$^+$ T cells expressing TCR Vα2 after secondary infection or immunisation is shown. The dashed line indicates the percentage of Vα2$^+$ cells seen in the naive donor cell population.

Values are the mean (± the SEM) of 3-8 mice per group per time point from 2-4 separate experiments.

5.8 Kinetics of High Avidity Vα2$^+$ FV-Specific CD4$^+$ T cells in the Absence of B cells

During FV infection, antigen can be presented by several types of specialised APCs resident in the spleen (the major site of FV infection), including dendritic cells, macrophages and B cells. It has been shown that after FV infection of B6-*Igh6*$^{-/-}$ mice which do not have any B cells, high avidity cells in the FV-specific CD4$^+$ T cell population were maintained (Ploquin et al., manuscript submitted). However, due to the absence of B cells, in this situation the spleen has an altered cell composition and architecture which results in an altered immune response and a more severe FV infection. To investigate the FV-specific CD4$^+$ T cell response in the absence of B cells without live viral infection, B6-*Igh6*$^{-/-}$ were immunised with env$_{124\text{-}138}$ peptide which, as shown in section **5.5.2**, is able to induce an FV-specific CD4$^+$ T cell response.

Immunisation of mice in the absence of B cells induced an FV-specific CD4$^+$ T cell response comparable to that seen after immunisation of complete B6 mice with a similar peak expansion and steady rate of contraction of FV-specific CD4$^+$ T cell numbers (**Figure 5.9A**). However, in accordance with results seen after FV-infection in the absence of B cells, high avidity FV-specific CD4$^+$ T cells were not lost as rapidly after expansion as in immunocompetent mice and by day 35 the FV-specific CD4$^+$ T cell population was comprised of a significantly higher proportion of high avidity cells ($p=<0.0001$) (**Figure 5.9B**).

These data further verified the observation that high avidity cells were maintained after infection of B6-*Igh6*$^{-/-}$ mice with FV, confirming that in the absence of B cells, high avidity FV-specific CD4^{+} T cells can be maintained. This suggested that deletion of high avidity clones in the FV-specific CD4^{+} T cell population was occurring as the result of interaction with B cells. This interaction was perhaps in place as a mechanism to regulate and therefore in the case of FV infection, limit virus-specific CD4^{+} T cell expansion.

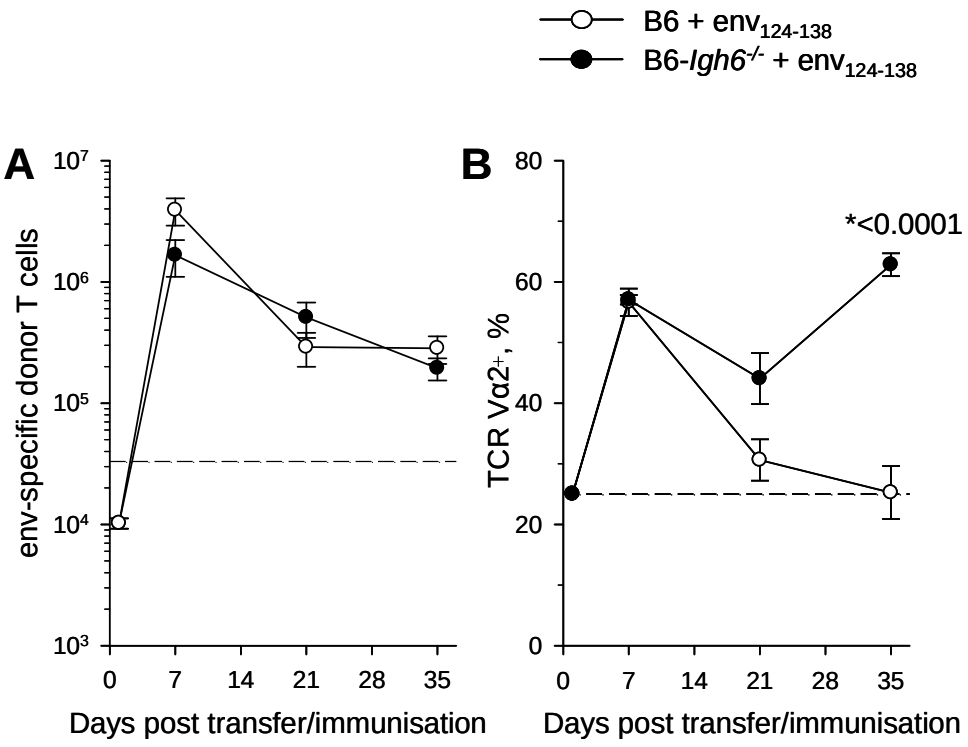

Figure 5.9 Kinetics and clonal composition of FV-specific TCRβ-transgenic CD4$^+$ T cells in B6-*Igh6*$^{-/-}$ mice after immunisation with env peptide

2×10^6 CD45.1$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 or B6-*Igh6*$^{-/-}$ mice (CD45.2$^+$) 1 day before immunisation with env$_{124\text{-}138}$ and adjuvant.

(A) Numbers of FV-specific donor CD4$^+$ T cells recovered after immunisation are shown. The dashed line indicates maximum donor cell expansion in the absence of infection.

(B) Percentage of FV-specific donor CD4$^+$ T cells expressing TCR Vα2 is shown. The dashed line indicates the percentage of Vα2$^+$ cells seen in the naive donor cell population. The number within the graph denotes the *p* value as compared using a two-tailed student's t-test.

Values are the mean (± the SEM) of 6-9 mice per group per time point from 2 separate experiments.

5.9 Discussion

5.9.1 Infection of $CD4^+$ T cells by FV does not contribute to their loss

HIV preferentially infects HIV-specific $CD4^+$ T cells, which are gradually deleted during HIV infection via mechanisms which are not currently understood (Douek et al., 2002). Although the major targets of FV infection are erythroid precursors and B cells, FV can infect any cell which expresses the ecotropic MuLV receptor CAT-1 and so $CD4^+$ T cells can theoretically be infected, and FV-specific T cells may be particularly susceptible. The initial question regarding the problem of high avidity FV-specific $CD4^+$ T cell loss was whether infection of $CD4^+$ T cells by FV was causing selective loss of high avidity FV-specific $CD4^+$ T cells in the FV model. With this in mind the *Fv1* allele was exploited, and loss of high avidity cells within a population which was genetically susceptible to infection was compared to that within cells that were not genetically susceptible to infection. There was no preservation of high avidity FV-specific $CD4^+$ T cells which were genetically not susceptible to FV infection was observed compared to those which were susceptible to FV infection. Whether FV-specific $CD4^+$ T cells are infected is not shown by these results, but it can be concluded that direct infection of virus-specific $CD4^+$ T cells does not contribute to the selective loss of high avidity FV-specific $CD4^+$ T cells in the FV model.

5.9.2 Differentiation level of high avidity FV-specific $CD4^+$ T cells

The model of progressive differentiation describes that different levels of antigen stimulation lead to different T cell cytokine profiles, and cells which receive very high levels of antigen stimulation are differentiated to a level whereby they undergo activation-induced cell deletion (Lanzavecchia and Sallusto, 2005). Intra-cellular cytokine staining demonstrated that high and low avidity FV-specific $CD4^+$ T cells contained IL-2 single producer, IL-2/IFN-γ double producer and IFN-γ single producer populations. Although there was no difference in the percentage of either IL-2 of IFN-γ single producers within the two populations, there were significantly more double producers in the high avidity FV-specific $CD4^+$ T cell population. This demonstrated that the high avidity FV-specific $CD4^+$ T cell population was not differentiated to a higher level than the low avidity FV-specific $CD4^+$ T cell population. However, it did show that more $CD4^+$ T cells in the high avidity FV-specific population were differentiated in general than in the low avidity population.

CD43 was also expressed at a significantly higher level on high avidity cells compared to low avidity cells at day 14 after FV infection, confirming that these high avidity cells were more activated than their low avidity counterparts. Parallel studies have demonstrated further differences in activation and differentiation between high and low avidity cells. These studies showed that compared to low avidity FV-specific $CD4^+$ T cells, high avidity FV-specific $CD4^+$ T cells demonstrated further characteristics of elevated activation, including reduced expression of the T cell inhibitory molecule programmed death 1 (PD-1), and increased expression of the T cell stimulatory molecule inducible costimulator (ICOS) and the signalling lymphocyte activation molecule (SLAM) Ly108 (Ploquin et al., manuscript

submitted). Together, these data showed that high avidity FV-specific $CD4^+$ T cells are activated more than their low avidity counterparts and for longer. This high level of prolonged activation of FV-specific $CD4^+$ T cells suggests that they are likely to be predisposed to AICD.

5.9.3 Homeostatic cytokine expression

Signals from homeostatic cytokines such as IL-2, IL-7 and IL-15 are required for the development and maintenance of memory T cells (Lanzavecchia and Sallusto, 2005). The hypothesis that high avidity FV-specific $CD4^+$ T cells may be unable to survive due to a compromised ability to receive signals from these homeostatic cytokines was investigated. When expression of receptors for these cytokines was compared on high and low avidity FV-specific $CD4^+$ T cells, no significant difference was seen in the proportion of cells expressing receptors for IL-7 and IL-15. However, the receptor for IL-2 was expressed on significantly more cells in the high avidity population at day 14 after infection than in the low avidity population.

These data suggest that the ability to receive more signals from IL-2 endows high avidity FV-specific $CD4^+$ T cells with a survival advantage over low avidity cells. However, IL-2 has been shown to have a negative effect on division of memory $CD8^+$ T cells, and this may also apply to $CD4^+$ T cells (Ku et al., 2000). The increased expression of IL-2R on high avidity FV-specific $CD4^+$ T cells may therefore potentially be detrimental to these cells, and could in fact contribute to their loss rather than enhancing their survival. It must also be considered that although statistical significance has been observed, because the difference in expression of IL-2R between high and low avidity cells is very low it is possible that it is not biologically significant, and hence does not affect survival of high avidity FV-specific $CD4^+$ T cells.

5.9.4 Persistent antigen and its contribution to the loss of high avidity FV-specific $CD4^+$ T cells

The observation that high avidity FV-specific $CD4^+$ T cells were lost during FV infection in immunocompetent mice is comparable to the $CD8^+$ T cell loss observed during chronic persistent viral infection (Moskophidis et al., 1993; Fuller et al., 2004). High avidity FV-specific $CD4^+$ T cells have been shown to be activated for longer and to contain a higher proportion of differentiated cells than the low avidity cell population, and FV infection has been shown to be persistent (Chesebro et al., 1979). In line with previous observations of T cell loss during chronic infection, the loss of high avidity $CD4^+$ T cells due to chronic antigenic stimulation during persistent FV infection was investigated. In order to test this hypothesis, experiments were carried out using non-persisting sources of antigen to induce an FV-specific immune response, specifically F-MuLV env peptide immunisation and immunisation with an F-MuLV-induced tumour cell-line, and the FV-specific $CD4^+$ T cell response was examined. The results presented showed that the overall FV-specific $CD4^+$ T cell population was lost at the same rate after induction with a non-persisting antigen source as when antigen was persisting. High avidity FV-specific $CD4^+$ T cells were preferentially induced but despite the absence of persisting antigen they were still preferentially deleted. Thus, it appears that persistent antigen stimulation of FV-specific $CD4^+$ T cells is not causing the loss of high avidity cells and therefore that non-persisting vaccination techniques may be unable to induce a virus-specific response dominated by high avidity $CD4^+$ T cells in some pathogens.

5.9.5 High levels of antigen do not cause high avidity $CD4^+$ T cell loss

In other experiments described here, low levels of antigen were unable to induce a long-lived FV-specific $CD4^+$ T cell response, while an increased level of antigen was able to induce a stronger overall FV-specific $CD4^+$ T cell response. Despite not causing loss of whole FV-specific $CD4^+$ T cells, a high level of antigen may have altered the clonal composition of the responding $CD4^+$ T cell population, and cause an exacerbated loss of high avidity $V\alpha2^+$ $CD4^+$ T cells, and a $CD4^+$ T cell response dominated by low avidity cells.

The relationship of antigen dose to clonal composition of virus-specific $CD4^+$ T cells was studied by employing the three variable antigen conditions described before. After infection with attenuated F-MuLV, where antigen level is low and the whole FV-specific $CD4^+$ T cell response has contracted by day 35 after infection, high avidity cells were lost even more rapidly, and had returned to the level seen in the naive $CD4^+$ T cell population sooner after infection than when antigen level was higher. Attenuated F-MuLV does not persist, and this further confirms the conclusion that high avidity FV-specific $CD4^+$ T cells are not exhausted and deleted due to persistent antigen stimulation.

At intermediate levels of antigen, using WT FV infection of B6 mice, high avidity cells are preferentially induced and subsequently lost, as described (Ploquin et al., manuscript submitted). In the condition where antigen level was at its highest, high avidity cells within the FV-specific $CD4^+$ T cell population were induced to higher levels. Notably, although they subsequently began to decline they did not reach the

levels seen in the naive $CD4^+$ T cell population or in those conditions where antigen was lower. Instead, high avidity cells began to recover and had gained a survival advantage over low avidity cells between days 21 and 35 after infection, suggesting that high antigen level is in fact beneficial to survival of high avidity FV-specific $CD4^+$ T cells, and that a high level of persistent antigen is not the cause of high avidity FV-specific $CD4^+$ T cell loss. These data further confirm that results observed regarding T cell loss during chronic persistent viral infections in previous studies, for example in LCMV, do not reflect the kinetics and the effect of high antigen level on the $CD4^+$ T cell response to FV. Potentially, the high avidity $V\alpha2^+$ cells present in the FV-specific $CD4^+$ T cell at day 35 after infection are of a different phenotype to those which expand at the peak of infection, and thus may be able to control chronic FV infection via different mechanisms compared to those employed by high avidity FV-specific $CD4^+$ T cells which control acute FV infection. This may explain the different roles of $CD4^+$ T cells observed at different stages of infection in this and previous studies which lead to the conclusion that $CD4^+$ T cells had a more important role during chronic infection than acute infection.

It has been shown that HAART-mediated prolonged suppression of HIV viraemia during HIV infection can lead to the loss of HIV-specific $CD4^+$ T cells (Pitcher et al., 1999). This supports the observation that lower antigen levels during a chronic and persistent retroviral infection are not sufficient to maintain a virus-specific memory $CD4^+$ T cell pool. Although FV infection is chronic and persistent, the level of antigen presented during a wild-type infection is apparently inadequate for maintenance of an FV-specific $CD4^+$ T cell response dominated by high avidity $CD4^+$ T cells.

5.9.6 Secondary immune responses to FV

The work shown here in section **3.2.1** demonstrates that immunisation of mice after transfer of EF4.1 TCRβ-transgenic $CD4^+$ T cells results in a memory FV-specific $CD4^+$ T cell response which is sufficient to reduce levels of FV infection in immunocompetent mice, to levels comparable to those seen when mice receive transgenic $CD4^+$ T cells in parallel with FV infection. The kinetics of the FV-specific $CD4^+$ T cell secondary response, particularly those of high avidity after FV infection or peptide immunisation were investigated. Secondary immunisation was able to induce a strong FV-specific $CD4^+$ T cell response and notably, high avidity cells within this $CD4^+$ T cell population were maintained at higher levels than that seen after primary peptide immunisation, or after wild-type infection. This suggests that secondary env peptide immunisation was able to boost the FV-specific $CD4^+$ T cell response, resulting in an FV-specific memory $CD4^+$ T cell pool that was dominated by high avidity cells.

In contrast, infection with FV 35 days after immunisation did not induce a strong overall FV-specific $CD4^+$ T cell secondary response. As mice have a reduced level of FV-infected cells in the spleen after peptide immunisation, the poor FV-specific $CD4^+$ T cell response observed during FV infection after peptide immunisation is likely to be due to the low level of antigen present. As seen in mice infected with attenuated FV, low levels of antigen are inadequate to induce a strong and long-lived FV-specific $CD4^+$ T cell response, explaining why control of FV infection after peptide immunisation results in a low level FV-specific $CD4^+$ T cell response.

5.9.7 Preservation of elevated levels of high avidity FV-specific CD4$^+$ T cells in the absence of B cells

Studies carried out in parallel to the work in this thesis have demonstrated that high avidity FV-specific CD4$^+$ T cells can be maintained after FV infection of mice deficient in B cells (Ploquin et al., manuscript submitted). However, due to lack of FV-specific antibodies in these mice infection is more severe. Disrupted splenic architecture and exacerbated FV infection in B-cell deficient mice may have altered the FV-specific CD4$^+$ T cell response, and the observation that high avidity FV-specific CD4$^+$ T cells can be maintained during infection of these mice may be a consequence of these factors. In order to overcome this limitation, mice deficient in B cells were immunised with peptide, which has been shown here to induce an FV-specific CD4$^+$ T cell response comparable to that of a WT FV infection. Although mice deficient in B cells still have altered splenic architecture, the absence of active virus infection allowed the clonal composition of the FV-specific CD4$^+$ T cell response to be more reliably assessed.

After peptide immunisation of B-cell deficient mice, the overall FV-specific CD4$^+$ T cell response declined at the same rate as that observed in a wild-type infection. Importantly, and in agreement with the parallel study, although high avidity FV-specific CD4$^+$ T cells within this population began to be deleted preferentially, they recovered and gained a survival advantage over low avidity cells, and by day 35 after infection the FV-specific CD4$^+$ T cell population was comprised of significantly more high avidity cells than control mice, with similar kinetics to those observed in FV infection of B6.A-*Fv2*^{s} mice where antigen is at a high level.

It is important to mention that despite an increased proportion of high avidity cells in the FV-specific $CD4^+$ T cell population at day 35 after FV infection where antigen is high, or peptide immunisation in B cell-deficient mice, an initial decline in high avidity cells was observed, Again, the resulting high avidity cells could potentially be of a different phenotype to those expanding during the peak of infection. The $CD4^+$ T cell kinetics observed after infection of B6.A-*Fv2*^{s} mice, including an increased level of high avidity FV-specific cells at day 35 after infection compared to day 35 after wild-type infection, are comparable to those seen after env peptide immunisation of B-cell-deficient mice, suggesting that an increased level of antigen is able to override the effect of B cell antigen presentation on the loss of high avidity $CD4^+$ T cells.

The data presented here and those from studies carried out in parallel suggest that an interaction between B cells and FV-specific $CD4^+$ T cells is contributing to the loss of high avidity cells with the overall population. The parallel studies also show that an FV-specific $CD4^+$ T cell population dominated by high avidity cells is evident when B-cell deficient mice were reconstituted with MHC class II-deficient B cells (Ploquin et al., manuscript submitted). This further suggests that an interaction taking place during presentation of antigen to high avidity FV-specific $CD4^+$ T cells by B cells leads to the preferential deletion of high avidity FV-specific $CD4^+$ T cell clones, in a potentially immunoregulatory manner.

Chapter Six

6 Effect of $CD4^+$ T cell Avidity on Protection Against FV Infection

6.1 Introduction

High avidity $CD8^+$ T cells have been shown to be more effective in clearing viral infections than those of a low avidity, and high avidity $CD4^+$ T cells are associated with resistance to Leishmaniasis (Malherbe et al., 2004; Sedlik et al., 2000). Furthermore, it is shown here that high avidity FV-specific $CD4^+$ T cells were activated for longer than their low avidity counterparts, and that there was a significantly higher proportion of double cytokine producers in the high avidity population. This suggested that the level of anti-viral activity demonstrated in section **3.2.2** mediated by high avidity FV-specific $CD4^+$ T cells would be more potent than that mediated by low avidity FV-specific $CD4^+$ T cells. As well as TCRα chain usage by low avidity FV-specific $CD4^+$ T cells, the anti-retroviral role of high avidity FV-specific $CD4^+$ T cells compared to low avidity FV-specific $CD4^+$ T cells was therefore examined.

In order to determine whether high avidity FV-specific $CD4^+$ T cell clones using the Vα2 TCR chain were more effective at reducing acute FV infection than those low avidity cells using alternative TCRα chains, $CD4^+$ T cells from B6.EF4.1 TCRβ-transgenic mice were purified and naive $V\alpha2^+$ and $V\alpha2^+$ cells were sorted and transferred separately into mice one day before FV infection. $V\alpha2^+$ and $V\alpha2^+$ cells were transferred in the ratio in which they were present ex vivo from B6.EF4.1 mice. For example, if 10% of the sorted whole $CD4^+$ T cell population were $V\alpha2^+$, mice would receive either 0.1×10^6 $V\alpha2^+$ cells or 0.9×10^6 $V\alpha2^-$ cells (ratio of 1:9).

6.2 TCR Vα Chain Usage by Low Avidity FV-specific $CD4^+$ T Cells

High avidity FV-specific $CD4^+$ T cells make a homogeneous $V\alpha2^+$ population, while Vα TCR chain usage by the low avidity virus-specific $CD4^+$ T cell population is not yet defined. To further characterise this population, PCR was employed to identify TCR Vα chain usage by low avidity $V\alpha2^-$ FV-specific donor $CD4^+$ T cells. $V\alpha2^-$ cells were sorted from three conditions

(i) B6 control mice

(ii) EF4.1 TCRβ-transgenic B6 mice

(iii) EF4.1 TCRβ-transgenic $CD4^+$ T cells stimulated with F-MuLV env peptide for 3 days *in vitro*

RNA was extracted from these sorted $V\alpha2^-$ cells, and synthesised cDNA was subjected to semi-quantitative PCR to identify Vα chain usage.

Results showed that Vα3 was expressed in unstimulated $V\alpha2^-$ TCRβ-transgenic $CD4^+$ T cells and at even higher levels in stimulated $V\alpha2^-$ TCRβ-transgenic $CD4^+$ T cells but not in B6 control $V\alpha2^-$ $CD4^+$ T cells. Vα8 was expressed weakly in *in vitro* peptide-stimulated $V\alpha2^-$ TCRβ-transgenic $CD4^+$ T cells but not in naive $V\alpha2^-$ TCRβ-transgenic $CD4^+$ T cells or B6 control cells. Vα4 was also expressed in both stimulated and unstimulated $V\alpha2^-$ TCRβ-transgenic $CD4^+$ T cells but has previously been identified as a non-productive transgene in B6.EF4.1 mice and thus is not indicative of Vα4 chain usage by low avidity $V\alpha2^-$ virus-specific $CD4^+$ T cells. Expression of other Vα TCR genes by cells from B6.EF4.1 mice was consistent with B6 control mice (**Figure 6.1**).

These data demonstrate that, corresponding with the high avidity FV-specific $CD4^+$ T cell population, the low avidity $V\alpha2^-$ virus-specific $CD4^+$ T cell population is also relatively homogeneous, with the transgenic FV-specific TCRβ chain preferentially pairing with the Vα3 and Vα8 endogenous TCRα chains. This provides a marker for further analysis of the low avidity FV-specific $CD4^+$ T cell population.

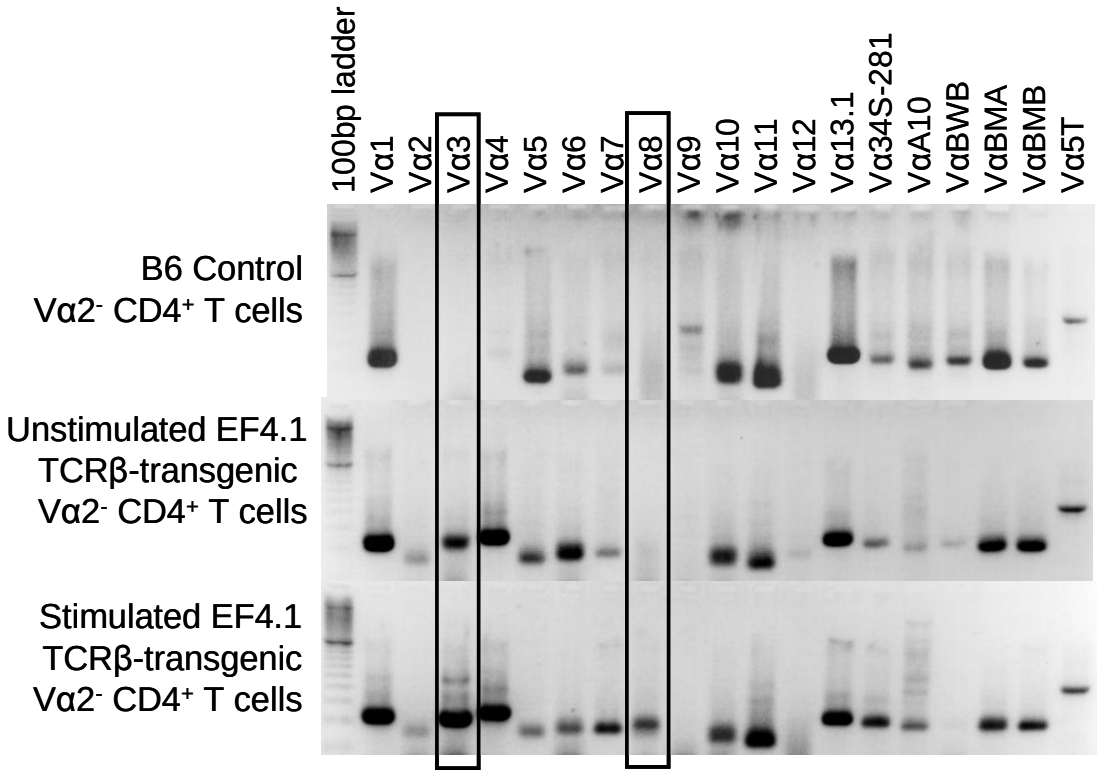

Figure 6.1 TCR Vα chain usage by Vα2$^-$ FV-specific CD4$^+$ T cells

Vα2$^-$ cells were sorted from naive B6 CD4$^+$ T cells, naive B6.EF4.1 CD4$^+$ T cells, and B6.EF4.1 TCRβ-transgenic CD4$^+$ T cells which had been stimulated *in vitro* with $env_{124-138}$ peptide for 72 hours. RNA was extracted and cDNA was synthesised and subjected to PCR using specific primers for Vα TCR chains.

6.3 Protection Against FV-induced Splenomegaly by High or Low Avidity $CD4^+$ T Cells in the Absence of $CD8^+$ T cells and B cells

As previously shown, FV-infected B6.A-*Fv2*^{s} *Rag1*$^{-/-}$ mice which received no $CD4^+$ T cells succumbed to splenomegaly by day 14 post infection. In contrast, mice which had received either $V\alpha2^+$ or $V\alpha2^-$ TCRβ-transgenic $CD4^+$ T cells maintained spleen indices typical of uninfected animals by day 14 (**Figure 6.2**). Other than one animal from the group which had received $V\alpha2^+$ TCRβ-transgenic $CD4^+$ T cells and which developed severe splenomegaly by day 21 post infection, all other mice were protected up to this point. However, we did observe symptoms of previously described immunopathology in mice which had received $V\alpha2^-$ TCRβ-transgenic $CD4^+$ T cells (Antunes et al., 2008).

These data show that adoptive transfer of either $V\alpha2^-$ or $V\alpha2^+$ TCRβ-transgenic $CD4^+$ T cells alone was sufficient to delay FV-induced splenomegaly in lymphopenic mice but that there was no protective advantage of high avidity $CD4^+$ T cells in the absence of other endogenous lymphocytes.

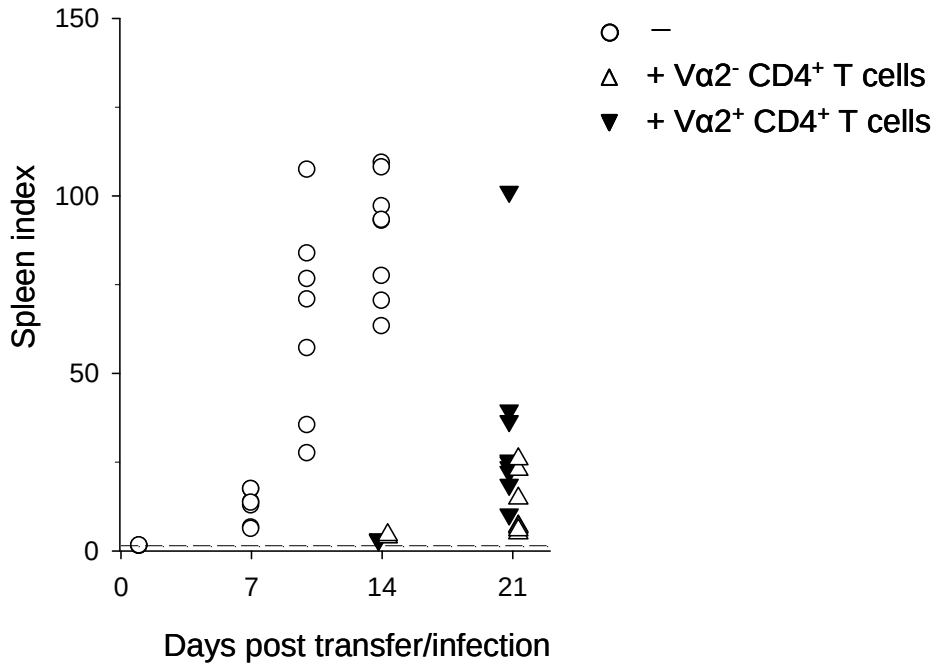

Figure 6.2 Protection against FV-induced splenomegaly in B6.A-*Fv2*^{s} *Rag1*$^{-/-}$ mice by high or low avidity EF4.1 TCRβ-transgenic CD4^{+} T cells

Spleen index after FV infection of B6.A-*Fv2*^{s} *Rag1*$^{-/-}$ mice without (-) or with (+ T cells) adoptive transfer of Vα2^{-} TCRβ-transgenic CD4^{+} T cells or Vα2^{+} TCRβ-transgenic CD4^{+} T cells is shown. Each symbol represents an individual mouse. The dashed line indicates the spleen index of uninfected mice.

6.4 Reduction of Acute FV Infection in Immunocompetent Mice by High or Low Avidity TCRβ-transgenic CD4$^+$ T Cells

Mice which had received whole TCRβ-transgenic CD4$^+$ T cells had significantly fewer infected cells than those which had received no CD4$^+$ T cells (p=0.0004), as shown in section **3.2.2**. Transfer of either Vα2$^+$, or Vα2$^-$ TCRβ-transgenic CD4$^+$ T cells alone was sufficient to reduce the percentage of infected cells in the spleen (p=0.01 and p=0.02 respectively), and this level of protection was not significantly different compared to mice which had received whole EF4.1 TCRβ-transgenic CD4$^+$ T cells. However, there was no significant difference in the levels of infected cells in the spleen when mice which received either Vα2$^+$ or Vα2$^-$ TCRβ-transgenic CD4$^+$ T cells were compared (**Figure 6.3**). These results showed that transfer of high avidity Vα2$^+$ or low avidity Vα2$^-$ clones individually were able to protect mice to a level comparable to that seen by transfer of whole TCRβ-transgenic CD4$^+$ T cells. However, there was no difference in the level of protection exerted by high avidity FV-specific CD4$^+$ T cells compared to low avidity FV-specific CD4$^+$ T cells at this early time point.

Further to this, Q-PCR was used to determine whether the level of F-MuLV in each of the groups was different during acute FV infection. DNA was extracted from spleen cells and Q-PCR was used to measure F-MuLV env DNA copies per 100,000 cells. No significant difference was seen between any of the groups when the two independent sample Wilcoxon Rank sum test was used to measure significance (**Figure 6.4**).

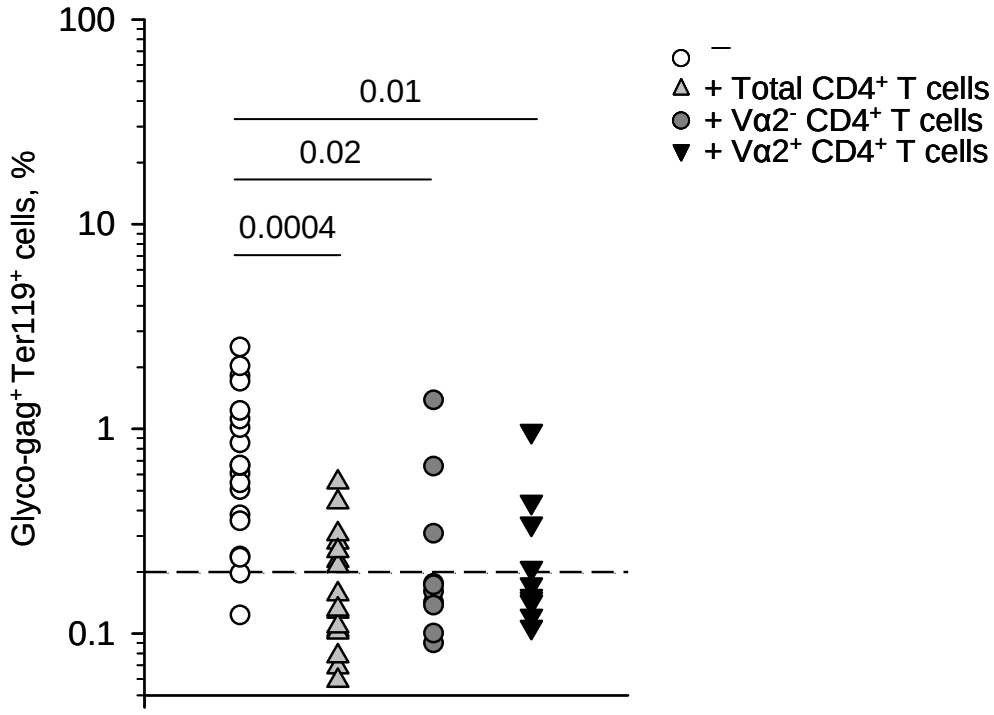

Figure 6.3 Protection against acute FV infection in B6 mice by additional high or low avidity EF4.1 TCRβ-transgenic CD4$^+$ T cells

Percentage of glyco-Gag$^+$Ter119$^+$ cells in the spleen of B6 mice, 7 days after FV infection, without (-) or with (+ T cells) adoptive transfer of whole TCRβ-transgenic CD4$^+$ T cells, Vα2$^-$ TCRβ-transgenic CD4$^+$ T cells or Vα2$^+$ TCRβ-transgenic CD4$^+$ T cells, 1 day before infection. Each symbol represents an individual mouse. The dashed line indicates the limit of flow cytometric detection. Numbers within the graph denote the *p* values as compared using two-tailed Wilcoxon-Mann-Whitney tests.

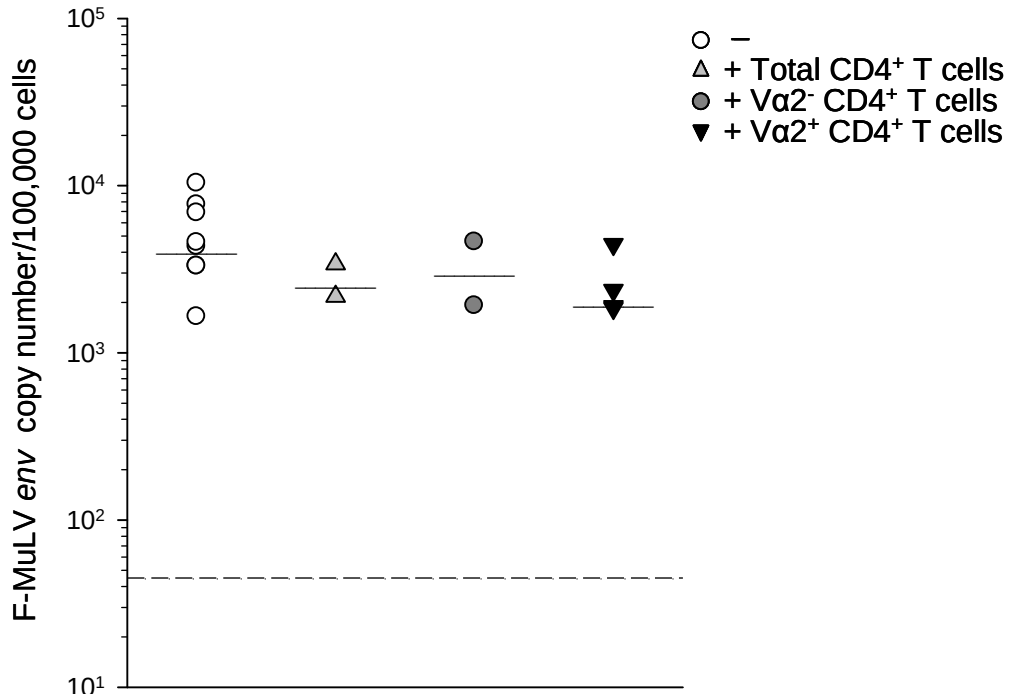

Figure 6.4 F-MuLV DNA during acute infection of B6 mice after adoptive transfer of high or low avidity EF4.1 TCRβ-transgenic CD4$^+$ T cells

Total TCRβ-transgenic CD4$^+$ T cells, Vα2$^-$ TCRβ-transgenic CD4$^+$ T cells or Vα2$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice which were FV infected 1 day later. DNA was extracted from spleens of mice 7 days after FV infection and Q-PCR was used to detect F-MuLV *env*. The copy number per 100,000 nucleated spleen cells is plotted. Each symbol represents an individual mouse. Solid lines represent the mean of each group. The dashed line indicates the numbers of copies of F-MuLV *env* per 100,000 cells in uninfected mice as detected by Q-PCR.

6.5 Control of Chronic FV infection in Immunocompetent Mice by High or Low Avidity EF4.1 TCRβ-transgenic CD4$^+$ T Cells

Q-PCR was also used to establish whether there was a difference in the level of FV infection after transfer of high or low avidity cells during the chronic phase of infection.

It was observed that in the group of animals which had received whole TCRβ-transgenic CD4$^+$ T cells, there was a trend towards fewer F-MuLV *env* DNA copies than in those which had received no cells but this was not statistically significant. In addition, mice which had received Vα2$^+$ TCRβ-transgenic CD4$^+$ T cells trended towards a lower amount of F-MuLV *env* DNA in the spleen than those which had received Vα2$^-$ TCRβ-transgenic CD4$^+$ T cells during the chronic phase, but once again this was not statistically significant (**Figure 6.5**).

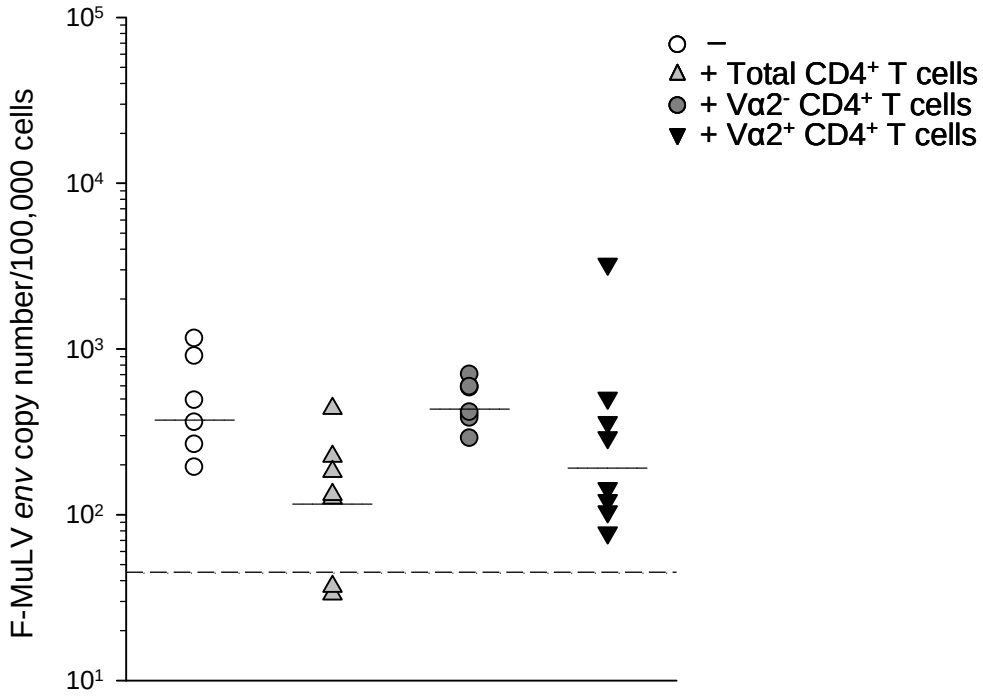

Figure 6.5 F-MuLV DNA during chronic infection of B6 mice after adoptive transfer of high or low avidity EF4.1 TCRβ-transgenic CD4$^+$ T cells

Total TCRβ-transgenic CD4$^+$ T cells, Vα2$^-$ TCRβ-transgenic CD4$^+$ T cells or Vα2$^+$ TCRβ-transgenic CD4$^+$ T cells were transferred into B6 mice which were FV infected 1 day later. DNA was extracted from spleens of mice 35 days after FV infection and Q-PCR was used to detect F-MuLV *env*. The copy number per 100,000 nucleated spleen cells is plotted. Each symbol represents an individual mouse. Solid lines represent the mean of each group. The dashed line indicates the numbers of copies of F-MuLV *env* per 100,000 cells in uninfected mice as detected by Q-PCR.

6.6 Discussion

6.6.1 Heterogeneity of low avidity FV-specific $CD4^+$ T cells

In order to establish TCR Vα chain use in the low avidity $V\alpha2^-$ FV-specific $CD4^+$ T cell population, PCR amplification of TCR Vα chains was used. Vα3 was identified as the preferred TCR Vα chain of the low avidity FV-specific $CD4^+$ T cell population. T cell hybridomas support this data and thus indicate that, similar to the high avidity population, the low avidity FV-specific $CD4^+$ T cell population is relatively homogeneous. The identification of the use of the Vα3 TCR chain by low avidity cells also provides a marker which may be used to identify and further investigate low avidity FV-specific $CD4^+$ T cells.

6.6.2 Effect of $CD4^+$ T cell avidity on protection

Previously, high avidity virus-specific $CD8^+$ T cells have been shown to be more protective than those of low avidity (Alexander-Miller et al., 1996; Sedlik et al., 2000). Furthermore, a $CD4^+$ T cell response dominated by high avidity cells correlated with resistance of mice against Leishmania infection (Malherbe et al., 2000). A high avidity clone using the Vα2 TCR chain has been identified within the EF4.1 FV-specific $CD4^+$ T cell population (Antunes et al., 2008). Further to this, the effect of $CD4^+$ T cell avidity on protection against FV infection was investigated here.

While transfer of high or low avidity EF4.1 TCRβ-transgenic $CD4^+$ T cells was able to reduce levels of FV infection comparable to that seen after transfer of whole EF4.1 TCRβ-transgenic $CD4^+$ T cells, transfer of high avidity $V\alpha2^+$ TCRβ-transgenic $CD4^+$

T cells did not result in increased protection compared to transfer of low avidity Vα2$^-$ TCRβ-transgenic CD4$^+$ T cells in immunocompetent mice. This experiment was repeated in mice with no endogenous adaptive immune cells and susceptibility at the *Fv2* allele. Transfer of either high or low avidity EF4.1 TCRβ-transgenic CD4$^+$ T cells was able to delay the onset of splenomegaly compared to mice which had received no CD4$^+$ T cells. However, mice which had received high avidity EF4.1 TCRβ-transgenic CD4$^+$ T cells began to develop large spleens at day 21 after infection, while those mice which had received low avidity cells did not.

Notably, mice which had received low avidity EF4.1 TCRβ-transgenic CD4$^+$ T cells began to show symptoms of disease which were not consistent with those observed in FV-induced disease, and upon further investigation no splenomegaly was observed in these animals. It has been previously demonstrated that an uncontrolled FV-specific CD4$^+$ T cell response can result in immunopathology manifested as anaemia (Antunes et al., 2008). Although no measure of red blood cell count was carried out in the current study, symptoms and spleen appearance were in line with those seen in CD4$^+$ T cell-induced anaemia in the previous study.

High or low avidity cells were transferred in the ratio in which they were extracted from mice, as described. Hence, the suspected and likely immunopathology is potentially due to an increased number of low avidity FV-specific CD4$^+$ T cells, rather than a difference in avidity. Together the results shown here, and in previous work by Antunes et al. suggest that a subtle balance of CD4$^+$ T cell avidity and cell

number must be achieved in order to induce adequate $CD4^+$ T cells to protect against FV infection while avoiding immunopathology.

Importantly, these results demonstrated that cells of both low and high avidities were induced during acute FV infection to a level at which they could contribute to control of FV, showing that there is sufficient antigen to stimulate FV-specific $CD4^+$ T cells regardless of avidity. Alternatively, low avidity cells may be required for protection during acute FV infection, while high avidity cells may play a role in the chronic phase of infection where the antigen level is low and potentially unable to be seen by $CD4^+$ T cells of low avidity. However it is possible that $CD4^+$ T cell avidity in fact does not determine the extent of anti-viral ability of FV-specific $CD4^+$ T cells, and further work is required in order to determine the exact effect of $CD4^+$ T cell avidity on protection against FV infection.

Chapter Seven

7 Future Work and Concluding Remarks

7.1 Future Work

In order to continue the study of $CD4^+$ T cells in retroviral infection, it is important to establish the mechanism by which $CD4^+$ T cells are exerting their anti-retroviral effect. $CD4^+$ T cell derived IFN-γ has been shown not to be required for control of acute FV infection. However, endogenous IFN-γ may act on adoptively transferred $CD4^+$ T cells to enhance their potential cytotoxic activity. Generation of EF4.1 TCRβ-transgenic mice which are deficient in the receptor for IFN-γ would further determine whether IFN-γ was acting on adoptively transferred $CD4^+$ T cells in order to enhance infected cell killing.

An additional potential and previously described mechanism of cytotoxicity mediated by $CD4^+$ T cells against FV is induction of the Fas/FasL pathway. Further investigation may reveal that $CD4^+$ T cells have multiple direct mechanisms which work in synergy to exert the maximum possible anti-retroviral effect.

It is shown here that peptide immunisation with a Th epitope is able to induce an FV-specific $CD4^+$ T cell response comparable to that of the wild-type infection, and that peptide immunisation in B cell-deficient mice is able to induce an FV-specific response dominated by high avidity $CD4^+$ T cells. Peptide immunisation using adjuvants which specifically agonise TLRs on APCs other than B cells, for example TLR-3 which is selectively expressed on DCs. Polyinosinic-polycytidylic acid (Poly I:C), is a TLR-3 agonist which could be used as an alternative to the Sigma adjuvant system during peptide immunisation with an F-MuLV env peptide, in order to promote antigen presentation by dendritic cells.

Identification of Vα3 chain usage by the low avidity Vα2$^-$ FV-specific CD4$^+$ T cell population provides a marker with which cells from the low avidity FV-specific CD4$^+$ T cell population can be identified and further examined for functionality, and for protective capacity compared to high avidity FV-specific CD4$^+$ T cell. Additionally, this low avidity population could be enriched in order to further examine the effect of T cell avidity on protection against FV infection.

7.2 Concluding Remarks

7.2.1 A novel role for $CD4^+$ T cells in control of acute FV infection

Previous studies investigating the role of $CD4^+$ T cells in immune responses against FV infection have been inconclusive, and from the data shown here it is clear that these previous results cannot be relied upon to give an accurate representation of the immune response to FV due to the caveats discussed above. As well as the likelihood of LDV contamination of FV stocks, it has not previously been possible to quantify the $CD4^+$ T cell response to FV. The use of clean FV stock and the EF4.1 TCRβ-transgenic mouse, which enables detection of the FV specific $CD4^+$ T cell response, has allowed a reassessment of the role of $CD4^+$ T cells against FV. Here, a previously unappreciated anti-retroviral role for $CD4^+$ T cells against FV infection has been demonstrated. The results here show that $CD4^+$ T cells play an important role in the control of acute FV infection, in contrast to results from previous studies which were unable to identify a role for $CD4^+$ T cells during the acute phase of FV infection. Findings from previous studies may have been due to LDV contamination of FV stock, or an inability to detect FV-specific $CD4^+$ T cells in response to infection, both of which are overcome in this study by the use of a clean FV stock and the EF4.1 TCRβ-transgenic mouse. Furthermore, these anti-retroviral $CD4^+$ T cells were shown to control FV infection independently of other adaptive immune cells, and their anti-retroviral effect was not mediated by IFN-γ, suggesting a direct anti-retroviral mechanism of $CD4^+$ T cells which has not been widely described in FV infection since the issue of LDV contamination was acknowledged. Further work potentially identifying direct $CD4^+$ T cell killing and the major mediators of this would be in agreement with observed *ex vivo* $CD4^+$ T cell cytotoxicity in HIV, further

consolidating the FV model as a viable model for the study of $CD4^+$ T cells in retroviral infection.

It is also shown here that virus-specific $CD4^+$ T cell expansion and contraction in the FV mouse model is different to that observed in other mouse models of viral infection using non retroviruses. This suggests that studying the $CD4^+$ T cell response to FV infection will provide more accurate information regarding the $CD4^+$ T cell response to retroviral infection, and hence is a potentially valuable mouse model for the study of immune responses to retroviral infections.

The results showing that $CD4^+$ T cells do play a role in controlling acute retroviral infection suggest that vaccination strategies targeted towards inducing retrovirus-specific $CD4^+$ T cells are valid potential candidates for a successful vaccine to protect against retroviral infection.

7.2.2 High avidity FV-specific CD4$^+$ T cells are lost due to previously undescribed mechanisms

Many factors which have previously been shown to contribute to the loss of T cells during viral infection and their effect on the loss of high avidity FV specific CD4$^+$ T cells have been investigated and discussed here. In summary, high avidity FV-specific CD4$^+$ T cells were not lost due to direct infection by the virus and did not have a reduced ability to receive signals from homeostatic cytokines. In spite of high avidity FV-specific CD4$^+$ T cells being activated for longer and having a higher proportion of double Th1 type cytokine producers than their low avidity counterparts, they were not lost due to exhaustion or terminal activation-induced deletion and were maintained in the absence of persistent antigen or chronic antigen stimulation. In contrast, a high level of persistent antigen favours their survival, and results in an FV-specific CD4$^+$ T cell response dominated by high avidity clones.

Notably, in accordance with previous data demonstrating the preservation of high avidity cells within the FV-specific CD4$^+$ T cell population during FV infection of B-cell deficient mice, high avidity cells were maintained after immunisation with an F-MuLV env peptide of the same mice, and continued to dominate the FV-specific CD4$^+$ T cell population despite no increase in the overall response. Prior work from the lab has shown that loss of high avidity CD4$^+$ T cells does not occur when B cells do not express MHC class II, showing that an interaction between B and T cells during antigen presentation by B cells to CD4$^+$ T cells is causing the preferential loss of high avidity FV-specific CD4$^+$ T cells. Furthermore, this loss of high-avidity cells did not occur during FV infection of mice deficient in the pro-apoptotic molecule SLAM-associated protein (SAP), which is required for SLAM signalling, correlating

with the observed increased expression of the SLAM Ly108 on high avidity FV-specific $CD4^+$ T cells compared to those of low avidity (Ploquin et al., manuscript submitted). This shows a previously unrecognised effect of B cells on shaping the clonal composition of a virus-specific T cell response. Although B cells have been shown to be required for efficient T cell development (Lund and Randall, 2010), their role in formation of a clonal response was not previously known.

The above described findings suggests that vaccine approaches which target APCs other than B cells for antigen presentation would be able to induce a retrovirus-specific $CD4^+$ T cell response dominated by high avidity $CD4^+$ T cells. There are several approaches by which this may be carried out. The first of these approaches is the use of TLR-specific peptide immunisation adjuvants, which selectively agonise TLRs expressed on DCs only and not B cells, which as described above can easily be tested in the FV mouse model. A recently trialled technique is that of targeting DC-SIGN (dendritic cell-specific intracellular adhesion molecule).

Vaccination studies have shown that targeting specific receptors on DCs results in induction of a broad adaptive virus-specific immune response, for example in EBV (Gurer et al., 2008). In HIV, a therapeutic vaccination technique where HIV-infected patients received autologous DCs loaded with HIV antigen demonstrated a reduction in viral load which correlated with virus-specific T cell responses. Together, these studies and the data shown here suggest that DC-targeted vaccination techniques provide an alternative vaccine candidate for induction of protection against retroviral infection.

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Appendices

Appendix I

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